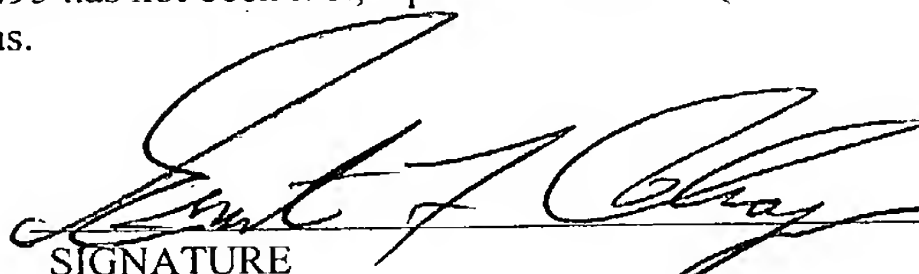


1007 Rec'd PCT/PTO 0 8 JAN 2002

FORM PTO-1390 REV. 2/01T		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 03806.0531	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37CFR1.5)	
				10/030230	
INTERNATIONAL APPLICATION NO. PCT/FR00/01937		INTERNATIONAL FILING DATE July 6, 2000		PRIORITY DATE CLAIMED July 8, 1999	
TITLE OF INVENTION METHOD FOR PURIFYING GRANULOCYTE COLONY STIMULATING FACTOR					
APPLICANT(S) FOR DO/EO/US Jacques DUMAS; Lucien REY; and Edoardo SARUBBI					
Applicant(s) herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1.	<input checked="" type="checkbox"/>	This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.			
2.	<input type="checkbox"/>	This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.			
3.	<input type="checkbox"/>	This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.			
4.	<input checked="" type="checkbox"/>	The US has been elected by the expiration of 19 months from the priority date (Article 31).			
5.	<input checked="" type="checkbox"/>	A copy of the International Application as filed (35 U.S.C. 371 (c)(2)).			
		a.	<input checked="" type="checkbox"/>	is attached hereto (required only if not communicated by the International Bureau).	
		b.	<input type="checkbox"/>	has been communicated by the International Bureau.	
		c.	<input type="checkbox"/>	is not required, as the application was filed with the United States Receiving Office (RO/US).	
6.	<input checked="" type="checkbox"/>	An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).			
		a.	<input checked="" type="checkbox"/>	is attached hereto (17 pages).	
		b.	<input type="checkbox"/>	has been previously submitted under 35 U.S.C. 154 (d)(4).	
7.	<input checked="" type="checkbox"/>	Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)).			
		a.	<input type="checkbox"/>	are attached hereto (required only if not communicated by the International Bureau).	
		b.	<input type="checkbox"/>	have been communicated by the International Bureau.	
		c.	<input type="checkbox"/>	have not been made; however, the time limit for making such amendments has NOT expired.	
		d.	<input checked="" type="checkbox"/>	have not been made and will not be made.	
8.	<input type="checkbox"/>	An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).			
9.	<input type="checkbox"/>	An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).			
10.	<input type="checkbox"/>	An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).			
Items 11 to 20 below concern document(s) or information included:					
11.	<input checked="" type="checkbox"/>	Information Disclosure Statement under 37 CFR 1.97 and 1.98			
12.	<input type="checkbox"/>	An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.			
13.	<input checked="" type="checkbox"/>	A FIRST preliminary amendment with Appendix and Abstract of the Disclosure.			
14.	<input type="checkbox"/>	A SECOND or SUBSEQUENT preliminary amendment.			
15.	<input checked="" type="checkbox"/>	A Substitute specification (17 pages).			
16.	<input type="checkbox"/>	A change of power of attorney and/or address letter.			
17.	<input type="checkbox"/>	A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.			
18.	<input type="checkbox"/>	A second copy of the published international application under 35 U.S.C. 154 (d)(4).			
19.	<input type="checkbox"/>	A second copy of the English language translation of the international application 35 U.S.C. 154 (d)(4).			
20.	<input checked="" type="checkbox"/>	Other items or information:			
		a.	<input checked="" type="checkbox"/>	Copy of cover page of International Publication No. WO01/04154 A1	
		b.	<input type="checkbox"/>	Copy of Notification of Missing Requirements.	
		c.	<input checked="" type="checkbox"/>	Marked-up specification (18 pages).	

U.S. APPLICATION NO. (If known, see 37CFR 1.5) 10/030230		INTERNATIONAL APPLICATION NO. PCT/FR00/01937		ATTORNEY'S DOCKET NUMBER 03806.0531	
21. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33 (1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				CALCULATIONS PTO USE ONLY	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	15	- 20 =	0	x \$18.00	\$
Independent Claims	2	- 3 =	0	x \$84.00	\$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+\$280.00	\$	
TOTAL OF THE ABOVE CALCULATIONS =				\$890.00	
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$	
SUBTOTAL =				\$890.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest priority date (37 CFR 1.492(f)).				\$	
TOTAL NATIONAL FEE =				890.00	
Fee for recording the enclosed assignment (37 CFR 1.21 (h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property.				\$	
TOTAL FEES ENCLOSED =				\$890.00	
				Amount to be refunded:	\$
				charged:	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>890.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>06-0916</u> . A duplicate copy of this sheet is enclosed. d. <input type="checkbox"/> Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO: Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P. 1300 I Street, N.W. Washington, D.C. 20005-3315					
DATED: January 8, 2002				<div style="text-align: center;">  SIGNATURE Ernest F. Chapman, Reg. No. 25,961 NAME/REGISTRATION NO. </div>	

JC13 Rec'd PCT/PTO 08 JAN 2002

PATENT

Attorney Docket No. 03806.0531

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. National Stage Application of
PCT/FR00/01937

Applicants:

Group Art Unit: Not Yet Assigned

Jacques DUMAS et al.

Examiner: Not Yet Assigned

Filed: January 8, 2002

For: METHOD FOR PURIFYING GRANULOCYTE COLONY STIMULATING FACTOR

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Before examining this application, please amend it as follows:

IN THE SPECIFICATION:

Please replace the originally filed specification with the attached Substitute Specification, which includes the claims as originally filed.

IN THE ABSTRACT:

Please insert the attached Abstract of the Disclosure after the claims.

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IN THE CLAIMS:

Please cancel claims 6 and 13 without prejudice to, or disclaimer of, the subject matter contained therein.

Please amend claims 1-5, 7-12, and 14-17 as follows:

1. (Amended) A process for purifying granulocyte colony-stimulating factor (G-CSF) from a biological sample, said process comprising:
 - a) reducing the volume of the biological sample containing G-CSF by hydrophobic-interaction chromatography to obtain a concentrated, desalted, and enriched fraction;
 - b) passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound to obtain a concentrated, desalted, and enriched fraction containing G-CSF; and
 - c) collecting the G-CSF.
2. (Amended) The process according to claim 1, in which the G-CSF collected has a purity of at least 90%.
3. (Amended) The process according to claim 1, in which the biological sample is a cell culture supernatant.

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4. (Amended) The process according to claim 1, in which the G-CSF is a human G-CSF (h G-CSF).

5. (Amended) The process according to claim 1, in which reducing the volume comprises a) placing the biological sample on a phenyl-type hydrophobic-interaction chromatography support under conditions wherein the G-CSF binds to the support, and b) eluting the G-CSF.

7. (Amended) The process according to claim 5, in which binding to the phenyl-type hydrophobic-interaction chromatography support is carried out in a buffer with an ionic strength comprised between 0 and 60 mSi, and elution is carried out by reducing the ionic strength or the salt concentration in the binding buffer.

8. (Amended) The process according to claim 5, in which the binding to the phenyl-type hydrophobic-interaction chromatography support is carried out in a buffer containing NaCl at a concentration between 0.1 and 1 M.

9. (Amended) The process according to claim 8, in which the binding to the phenyl-type hydrophobic-interaction chromatography support is carried out in a buffer containing NaCl at a concentration between 0.1 and 0.5 M, and the elution is carried out with water.

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10. (Amended) The process according to claim 1, in which the passing of the concentrated fraction over hydroxyapatite is carried out in a buffer of ionic strength between 2 and 30 mSi and at a pH between 5.5 and 7.5.

11. (Amended) The process according to claim 10, in which the buffer comprises phosphate at a concentration between 1 and 10 mM.

12. (Amended) The process according to claim 10, in which the buffer is a 1 mM phosphate buffer and the pH is between 6.0 and 7.5.

14. (Amended) The process according to claim 1, further comprising purification by ion exchange chromatography, reversed phase gel filtration chromatography, affinity chromatography, or a combination of one or more of these types of purification steps.

15. (Amended) A process for eliminating contaminating proteins from a solution containing G-CSF, said process comprising

- a) passing the solution over hydroxyapatite under conditions where the contaminating proteins and the G-CSF bind to the hydroxyapatite, wherein the G-CSF is weakly bound, and
- b) eluting the G-CSF.

16. (Amended) The process according to claim 15, in which the G-CSF is eluted by washing with buffer used for binding the G-CSF.

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17. (Amended) The process according to claim 15, in which the solution containing the G-CSF is prepared by hydrophobic-interaction chromatography of a biological sample containing G-CSF.

REMARKS

By this Preliminary Amendment, a Substitute Specification (along with a marked-up copy indicating the changes made to the original specification) and an Abstract of the Disclosure are submitted, claims 6 and 13 are cancelled, and claims 1-5, 7-12, and 14-17 are amended.

The Substitute Specification is submitted in accordance with 37 C.F.R. § 1.125 in order to properly recite trademarks, to correct typographical and grammatical errors in the original specification, and to improve the readability of the application. A marked-up copy of the specification is also attached, showing the changes that have been made to the original specification, filed as part of the PCT application. No new matter has been added by the Substitute Specification.

An Abstract of the Disclosure, which merely summarizes the disclosure of the application, is attached. No new matter is entered by the new Abstract of the Disclosure.

The claims are amended to place them in proper format for examination in the U.S., and to correct typographical and grammatical errors. All of the changes to the claims find support in the claims as originally filed as International Application PCT/FR00/01937 on July 6, 2000.

Accordingly, no new matter is entered by the claim amendments.

Upon entry of this Preliminary Amendment, claims 1-5, 7-12 and 14-17 will be pending in this application.

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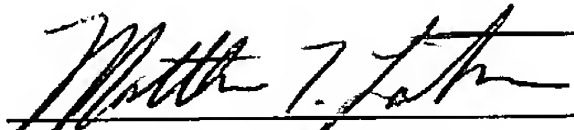
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Attorney Docket No. 03806.0351

Applicants believe that no fees are due in connection with the filing of this Preliminary Amendment. However, if any fees are due, please charge the fees to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

By: 
Matthew T. Latimer
Reg. No. 44,024
(202) 408-4495
matthew.latimer@finnegan.com

Date: January 8, 2002

Attachments:

Substitute Specification
Marked-up copy of Original Specification
Appendix

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APPENDIX

Accompanying Preliminary Amendment for Attorney Docket No. 03806.0531

IN THE CLAIMS:

Please amend claims 1-5, 7-12, and 14-17 as follows:

1.[] (Amended) [Process] A process for purifying granulocyte colony-stimulating factor (G-CSF) from a biological sample, said process comprising: [the stages of]

a) reducing the volume of the biological sample containing G-CSF by hydrophobic-interaction chromatography [in order] to obtain a concentrated, desalted, and enriched fraction;[,]

b) passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound [in order] to obtain a concentrated, desalted, and enriched fraction containing G-CSF; and

c) collecting the G-CSF.

2.[] (Amended) [Process] The process according to claim 1, in which the G-CSF collected has a purity of at least 90 %.

3.[] (Amended) [Process] The process according to claim 1, in which the biological sample is a cell culture supernatant.

4.[] (Amended) [Process] The process according to claim 1, in which the G-CSF is a human G-CSF (h G-CSF).

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5.[D)] (Amended) [Process] The process according to claim 1, in which reducing the volume [reduction stage] comprises a) placing [of] the biological sample on a phenyl-type hydrophobic-interaction chromatography support [of phenyl type] under conditions [allowing the fixation of] wherein the G-CSF binds to the support, and b) eluting the G-CSF [then its elution].

7.[D)] (Amended) [Process] The process according to claim [6] 5, in which [fixation on Phenyl Sepharose®] binding to the phenyl-type hydrophobic-interaction chromatography support is carried out in a buffer with an ionic strength comprised between 0 and 60 mSi, and elution is carried out by reducing the ionic strength or the salt concentration in the [fixation] binding buffer.

8.[D)] (Amended) [Process] The process according to claim [6] 5, in which the [fixation on Phenyl Sepharose®] binding to the phenyl-type hydrophobic-interaction chromatography support is carried out in a buffer containing NaCl at a concentration [comprised] between 0.1 and 1 M.

9.[D)] (Amended) [Process] The process according to claim 8, in which the [fixation on Phenyl Sepharose®] binding to the phenyl-type hydrophobic-interaction chromatography support is carried out in a buffer containing NaCl at a concentration [comprised] between 0.1 and 0.5 M, and the elution is carried out with water.

10.[]] (Amended) [Process] The process according to claim 1, in which the [stage of] passing of the concentrated fraction over hydroxyapatite is carried out in a buffer of ionic strength [comprised] between 2 and 30 mSi and at a pH [comprised] between 5.5 and 7.5.

11.[]] (Amended) [Process] The process according to claim 10, in which the buffer comprises phosphate at a concentration [comprised] between 1 and 10 mM.

12.[]] (Amended) [Process] The process according to claim 10, in which the buffer is a 1 mM phosphate buffer and the pH is [comprised] between 6.0 and 7.5.

14.[]] (Amended) [Process] The process according to claim 1, [13 in which the multistage process moreover comprises] further comprising purification by [one or more stages of chromatography chosen from the group constituted by] ion exchange chromatography, reversed phase gel filtration chromatography, [or] affinity chromatography, or a combination of one or more of these types of purification steps.

15.[]] (Amended) [Process] A process for eliminating [the] contaminating proteins from a solution containing G-CSF, said process [and the contaminating proteins] comprising

a) passing the solution over hydroxyapatite under conditions where [by which] the contaminating proteins and the G-CSF bind [are fixed] to the hydroxyapatite, wherein [and] the G-CSF is weakly bound, and

b) [elution of] eluting the G-CSF.

16.[.]) (Amended) [Process] The process according to claim 15, in which the [elution of the] G-CSF is eluted [carried out] by [simple] washing with [the fixation] buffer used for binding the G-CSF.

17.]]] (Amended) [Process] The process according to claim 15, in which the solution containing the G-CSF is prepared by hydrophobic-interaction chromatography of a biological sample containing G-CSF.

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Process for purifying granulocyte colony stimulating factor.

[0001] **BACKGROUND OF THE INVENTION AND RELEVANT ART**

[0002] The present invention relates to a process for purifying granulocyte colony stimulating factor (called G-CSF) by chromatography using a stage of chromatography on hydroxyapatite.

[0003] Among the colony stimulating factors which regulate the differentiation and the proliferation of mammalian hematopoietic cells, granulocyte colony stimulating factors have been described, for example in International Patent Application WO 87/01132 or European Patent Application EP169566.

[0004] The preparation of G-CSFs from different origins and their purification has been described in numerous scientific publications or patent applications. For example, European Patent Application EP 243153 described a process for purifying human G-CSF from cell lines of bladder carcinoma HBT5637; European Patent Application EP215126 described the purification of recombinant human G-CSF produced in *E. coli*. The processes described above correspond to multiple stages of purification in which the initial concentration of the starting biological preparations is generally obtained by standard methods of ultrafiltration or precipitation by a salt, followed by successive stages of reversed phase liquid chromatography (called RP-HPLC), which has the known drawback of leading to a significant loss of yield, for example because the protein is denatured by the organic solvents. Moreover, the American Patent US 5,055,555 described a selective and simplified process for purifying recombinant human G-CSF produced in a yeast on a larger scale, by precipitation by NaCl preceded by concentration by chromatography on a cation ion exchange column (S SEPHAROSE® or MONO S® (Pharmacia)), but of which the yield and the purity obtained are not mentioned.

[0005] In addition, several uses of chromatography stages, other than RP-HPLC, have also been described for purifying G-CSFs:

[0006] The use of PHENYL SEPHAROSE® CL-6B (Pharmacia) was described by N A. Nicola et al., Journal of Biological Chemistry, Vol. 258, p. 9017-9023, 1983 for purifying G-CSF produced naturally by murine leukaemia cells. After preliminary concentration of the

medium on hollow fibre and "salting out" chromatography, the G-CSF was directly fixed on the PHENYL SEPHAROSE® (Pharmacia) column, then eluted using a decreasing salt gradient, then a linear gradient of ethylene glycol.

[0007] The use of hydroxyapatite was described by T. Arakawa et al., Archives of Biochemistry and Biophysics, Vol. 316, p. 285-289, 1995 as a last stage of purifying G-CSF produced from transformed CHO cells.

[0008] The use of SP SEPHAROSE® Fast Flow (Pharmacia) was described by S-H Kang et al., Biotechnology Letters, Vol. 17, p. 687-692, 1995 for purifying G-CSF produced from transformed *E. coli* cells. After solubilization of the inclusion bodies and renaturation, the G-CSF was eluted using an NaCl gradient ranging from 0 to 0.5 M.

[0009]

DESCRIPTION OF THE INVENTION

[0010] One of the subjects of the present invention is to provide a process which allows the isolation and purification of G-CSFs, on a large scale and with high yields, by a stage of chromatography on hydroxyapatite from biological samples previously concentrated and enriched using hydrophobic-interaction chromatography.

[0011] The process of the invention can be used, for example, as a first stage of purifying G-CSF in a multistage process for the preparation of a G-CSF having a purity suitable for clinical use.

[0012] A subject of the invention is a process for purifying G-CSF from a biological sample comprising the stages of

- a) reducing the volume of the biological sample containing the G-CSF by hydrophobic-interaction chromatography in order to obtain a concentrated, desalted, and enriched fraction,
- b) passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound in order to obtain a concentrated, desalted, and enriched fraction containing G-CSF, and
- c) collecting the G-CSF.

[0013] The above process allows the G-CSF to be purified under non-denaturing conditions and to isolate the biologically active G-CSF.

[0017] In a quite unexpected and advantageous manner, the process according to the invention comprises a hydrophobic-interaction chromatography characterized by fixation of the protein at low conductivity or at a low salt content, for example ammonium sulphate or NaCl, and allows the volume of the initial biological sample to be reduced whilst eliminating the salts as well as a high percentage of the contaminating proteins. The process of the invention therefore allows a concentrated, desalted, and enriched in non-denatured G-CSF fraction to be obtained which is then passed over hydroxyapatite.

[0018] Contrary to the use of hydroxyapatite as a final stage of purification described by T. Arakawa et al., 1995 above, in which the G-CSF is collected in the unfixed fraction on the hydroxyapatite equilibrated in a 10 mM phosphate buffer containing 0.1 M NaCl at pH 7.0, the process according to the invention uses conditions where the G-CSF is weakly bound to the hydroxyapatite and thus allows a concentrated and desalted solution of purified G-CSF to be collected.

[0019] A particular subject of the invention is the above process in which the G-CSF collected has a purity of at least 90%.

[0020] A particular subject of the invention is also the above process in which the biological sample is a cell culture supernatant as well as the process in which the G-CSF is a human G-CSF (called hG-CSF).

[0021] A particular subject of the invention is also the above process in which the volume reduction stage comprises the placing of the biological sample on a hydrophobic-interaction chromatography support of phenyl type under conditions allowing the fixation of the G-CSF, then its elution.

[0022] A more particular subject of the invention is the above process in which the support of phenyl type is PHENYL SEPHAROSE® (Pharmacia).

[0023] A quite particular subject of the invention is the above process in which fixation on PHENYL SEPHAROSE® (Pharmacia) is carried out in a buffer with an ionic strength comprised between 0 and 60 mSi, and elution is carried out by reducing the ionic strength or the concentration of salt in the fixation buffer.

[0024] A more particular subject of the invention is also the above process in which fixation on PHENYL SEPHAROSE® (Pharmacia) is carried out in a buffer containing NaCl at a concentration comprised between 0.1 and 1 M.

[0025] A quite particular subject of the invention is the above process in which the fixation on PHENYL SEPHAROSE® (Pharmacia) is carried out in a buffer containing NaCl at a concentration comprised between 0.1 and 0.5 M, and elution is carried out using water.

[0026] Examples of using hydrophobic-interaction chromatography on PHENYL SEPHAROSE® (Pharmacia) illustrating the process according to the invention are described further on in the experimental part.

[0027] A particular subject of the invention is also the above process according to the invention in which the stage of passing over hydroxyapatite is carried out in a buffer with an ionic strength comprised between 2 and 30 mSi and at a pH comprised between 5.5 and 7.5.

[0028] A more particular subject of the invention is the above process in which the buffer comprises phosphate at a concentration comprised between 1 and 10 mM.

[0029] A more particular subject of the invention is also the above process in which the buffer is a 1 mM phosphate buffer and the pH is comprised between 6.0 and 7.5.

[0030] Examples of using hydroxyapatite following chromatography on PHENYL SEPHAROSE® (Pharmacia) illustrating the process according to the invention are described further on in the experimental part.

[0031] The invention also relates to a process of purifying G-CSF which can be included in a multistage process for purifying G-CSF from a biological sample comprising the stages of

- a) reducing the volume of the biological sample containing the G-CSF by hydrophobic-interaction chromatography in order to obtain a concentrated, desalted, and enriched fraction,
- b) passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound in order to obtain a concentrated, desalted, and enriched fraction containing the G-CSF, and
- c) collecting the G-CSF.

[0040] **Analytical methods**

[0041] 1. Assay of G-CSF by HPLC

[0042] The fractions collected after chromatography were analyzed by analytical RP-HPLC on a Vydac C4 column (0.46 x 15) , 300Å, 5 microns, equilibrated in H₂O/TFA 0.1%, at a flow rate of 2 ml/min with a linear gradient of acetonitrile/TFA 0.1% varying from 40 to 80% over 10 minutes, and spectrophotometric detection at 214 nm.

[0043] The G-CSF was eluted at a concentration of approximately 65% acetonitrile. The G-CSF concentration was measured by comparison with a G-CSF standard. Evaluation of the purity was measured by the ratio of the area of the G-CSF peak and the area of all peaks other than that of the injection peak.

[0044] 2. Assay of G-CSF by ELISA

[0045] The G-CSF concentration was measured by using the ELISA kit from R&D System Inc. and the protocol recommended by the supplier.

[0046] 3. SDS-PAGE

[0047] The samples were analyzed on ready-to-use polyacrylamide gels (Novex) containing a gradient of 10 to 20% polyacrylamide and a silver stain using the Biorad silver staining kit for a deposit of 50 ng to 1 µg of G-CSF.

[0048] **BRIEF DESCRIPTION OF THE FIGURES**

[0049] The attached figures illustrate certain aspects of the invention.

[0050] Figure 1 is a chromatogram showing the fractionation on PHENYL SEPHAROSE[®] (Pharmacia) of a supernatant of cells expressing GA-GCSF and containing 0.1 M NaCl. The arbitrary units represent the conductivity and the optical density (OD) respectively of the column effluent expressed as a percentage.

[0051] Figure 2 is a chromatogram showing the fractionation on PHENYL SEPHAROSE[®] (Pharmacia) of a supernatant of cells expressing GA-GCSF and containing 0.5 M NaCl. The arbitrary units have the same meaning as in Figure 1.

[0052] Figure 3 is a chromatogram showing the fractionation of GA-GCSF on hydroxyapatite MACROPREP[®] (BioRad) ceramic Type I after PHENYL SEPHAROSE[®] (Pharmacia). The arbitrary units have the same meaning as in Figure 1.

[0053] Figure 4 is a chromatogram of analytical RP-HPLC of GA-GCSF after PHENYL SEPHAROSE® (Pharmacia) and hydroxyapatite Type I.

[0054] Figure 5 is a chromatogram showing the fractionation of GA-GCSF on hydroxyapatite MACROPREP® (BioRad) ceramic Type II after PHENYL SEPHAROSE® (Pharmacia). The arbitrary units have the same meaning as in Figure 1.

[0055] Figure 6 is a chromatogram of analytical RP-HPLC of GA-GCSF after PHENYL SEPHAROSE® (Pharmacia) and hydroxyapatite Type II.

[0056] Figure 7 represents analysis by SDS-PAGE of the successive purification of GA-GCSF in a supernatant of filtered culture (well 3), an eluate of PHENYL SEPHAROSE® (Pharmacia) (well 4), an eluate of hydroxyapatite (well 5), an eluate of SP SEPHAROSE® (Pharmacia) (well 6), a UF concentrate (well 7), an eluate of filtration gel in PBS buffer (well 9), and an eluate of filtration gel in acetate buffer pH 5.5 (well 11), with standard molecular weight markers (well 1). The band corresponding to the apparent MW of the GA-GCSF is indicated by an arrow.

[0057] **EXAMPLES**

[0058] **Example 1:** Concentration of a biological sample of G-CSF by chromatography on PHENYL SEPHAROSE® (Pharmacia).

[0059] The starting material was the centrifugation supernatant of a culture broth of human cell lines expressing a human GA-GCSF obtained according to the International Patent Application WO95/31560 in an ENDOTRONICS® (Minneapolis, MN) hollow fibre bioreactor in DMEM/F12 (Hyclone) medium containing 0.9% foetal calf serum. After centrifugation, the supernatant was stored at -20°C before use.

[0060] The thawed supernatant was chromatographed on PHENYL SEPHAROSE® (Pharmacia) after adding 0.1 M NaCl q.s. and filtration on a 0.22 µm Millipore membrane, at a temperature of approximately 15 to 20°C.

[0061] Using a Pharmacia XK16 column (1.6 cm x 40 cm) provided with 50 ml of PHENYL SEPHAROSE® (Pharmacia) Fast Flow High Substitution (Pharmacia), stored under ethanol at 25%, then washed with Milli-Q de-mineralized water before use, then equilibrated with a 0.1M solution of NaCl, concentration by chromatography on PHENYL

SEPHAROSE® (Pharmacia) was carried out in the following manner: 2295 ml of salted and filtered supernatant obtained above (conductivity 17.7 mS.cm⁻¹) was applied to the column at a flow rate of 13 ml/min, and the effluent of the column was collected in fractions of 500 ml. The column was then washed at a flow rate of 4 ml/min with 220 ml of a 0.05 M NaCl solution, and the effluent of the column was collected in fractions of 40 ml. The column was then eluted at the same flow rate with 150 ml of Milli-Q demineralized water, and the effluent from the column was collected in fractions of 2 ml. The column was finally regenerated by washing at the same flow rate with an 8 M solution of urea.

[0062] The total proteins in the effluent of the column were detected by absorption at 280 nm and the salt concentration was monitored using a conductimeter. The presence in the column effluent of a first peak of protein eluted by water, then of a second peak of protein eluted by washing with urea is shown in Figure 1.

[0063] The fractions collected during elution with water were analyzed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA using the conditions described previously. The fractions containing the combined G-CSF (40 ml) contained 29.3 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 56% and a purity of 58%.

[0064] The solution of GA-GCSF PHENYL SEPHAROSE® (Pharmacia) thus obtained had a conductivity of 0.161 mS.cm⁻¹.

[0065] **Example 2:** Chromatography on PHENYL SEPHAROSE® (Pharmacia), then on hydroxyapatite as a first stage for purifying G-CSF.

[0066] The starting material was the supernatant of a culture broth of human cell lines expressing a human GA-GCSF obtained as in Example 1 but using a 5 litre bioreactor in the place of an ENDOTRONICS® (Minneapolis, MN) bioreactor. The thawed supernatant was chromatographed on PHENYL SEPHAROSE® (Pharmacia) after the addition of 0.5 M NaCl q.s. and filtration on a 0.45 µm Millipore membrane.

[0067] By using a Pharmacia XK16 column provided with 50 ml of PHENYL SEPHAROSE® (Pharmacia) Fast Flow High Substitution (Pharmacia) stored under ethanol at 25%, then washing with Milli-Q demineralized water before use, concentration by chromatography on PHENYL SEPHAROSE® (Pharmacia) was carried out in the following manner: 1640 ml of salted and filtered supernatant obtained above (conductivity

56.3 mS.cm⁻¹) was applied to the column at the flow rate of 4 ml/min and the effluent of the column was collected in fractions of 400 ml. The column was then eluted at the same flow rate of 4 ml/min with 240 ml of 0.5M NaCl, and the effluent of the column was collected in fractions of 40 ml. The column was then eluted at the same flow rate with 150 ml of Milli-Q de-mineralized water, and the the effluent of the column was collected in fractions of 2 ml. The column was finally regenerated by washing with the same flow rate with a solution of 8M urea.

[0068] The total proteins in the column effluent and the salt concentration were detected as in Example 1. The presence in the column effluent of a first peak of the protein eluted by washing with water, then of a second peak of the protein eluted by washing with urea is shown in Figure 2.

[0069] The fractions collected during elution with water were analyzed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA. The combined fractions containing the combined G-CSF (50 ml) contained 45.1 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 90% with a purity of 61%.

[0070] The solution of GA-GCSF PHENYL SEPHAROSE[®] (Pharmacia) thus obtained had a conductivity of 4.22 mS.cm⁻¹, which allowed it to be used as it is in the following stage of chromatography on hydroxyapatite.

[0071] Using a Pharmacia XK16 column provided with 29 g of hydroxyapatite MACROPREP[®] Ceramic, Type I (Bio-Rad), previously suspended in 250 mM sodium phosphate buffer at pH 7.3 (250 mM buffer, pH 7.3), then equilibrated by percolation with a flow rate of 5 ml/min of 500 ml of the 250 mM buffer diluted to 1/250 (1 mM buffer, pH 7.3), chromatography on hydroxyapatite was carried out in the following manner: 24 ml of the solution of GA-GCSF PHENYL SEPHAROSE[®] (Pharmacia) obtained above, then stored overnight at +2°C, was applied to the hydroxyapatite column at the same-flow-rate. The column was then eluted with 150 ml of 1 mM buffer, pH 7.3, then regenerated by washing with 250 mM buffer, pH 7.3 at the same flow rate, and the column effluent was collected in fractions of 5ml. The total proteins in the column effluent and the salt concentration were detected as in Example 2.

[0080] By using a Pharmacia XK 50/30 column provided with 500 ml of PHENYL SEPHAROSE® (Pharmacia) Fast Flow High Substitution (Pharmacia) stored under ethanol at 25%, then equilibrated with a solution of 0.5 M NaCl before use, concentration by chromatography on PHENYL SEPHAROSE® (Pharmacia) was carried out in the following manner: the concentrated salted supernatant obtained above (conductivity 40 mS.cm⁻¹) was applied to the column at a flow rate of 40 ml/min. The column was then successively eluted at the same flow rate with 1.5 litres of 0.5 M NaCl, then with 1.5 litres of Milli-Q water, and the column effluent was collected in fractions of 20 ml. The total proteins and the concentration of salts were detected as in Example 1.

[0081] The fractions corresponding to the peak of the protein eluted with water were analyzed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA. The combined fractions containing the G-CSF (400 ml) contained 357 mg of GA-CSF titrated by HPLC, corresponding to a yield of 67.9% with a purity of 20.6%. The solution of GA-GCSF PHENYL SEPHAROSE® (Pharmacia) thus obtained had a conductivity of 2 mS.cm⁻¹ at 20°C.

[0082] The solution of PHENYL SEPHAROSE® (Pharmacia) was also analyzed by SDS-PAGE (Figure 7, well 4). After stabilization by adding Pefabloc (0.2 mg/ml) and benzamidine (1mM), the solution was immediately used for the following stage of chromatography on hydroxyapatite.

[0083] By using a Pharmacia XK 50/30 column provided with 290 g of hydroxyapatite MACROPREP® Ceramic, Type II (Bio-Rad) previously suspended in 5 litres of 1 mM sodium phosphate buffer at pH 6 (1 mM buffer, pH 6), then equilibrated by percolation of 1 litre of 250 mM sodium phosphate buffer at pH 6 (250 mM buffer, pH 6) at a flow rate of 50 ml/min, then by percolation of 5 litres of 1 mM buffer, pH 6 (thus generating a column of 500 ml of hydroxyapatite), chromatography on hydroxyapatite was carried out in the following manner: 400 ml of the solution of CA-GCSF of stabilized PHENYL SEPHAROSE® (Pharmacia) obtained above was applied to the column of hydroxyapatite at a flow rate of 50 ml/min. The column was then eluted with 1.50 litres of 1 mM buffer, pH 6, collecting the column effluent in fractions of 50 ml. The total proteins as well as the conductivity of the column effluent were detected as indicated in Example 1.

[0084] The fractions collected were analyzed by analytical RP-HPLC and by ELISA. The combined fractions (400 ml) contained 331 mg of GA-GCSF titrated by HPLC, corresponding to a chromatography yield of 92.5% with a purity higher than the 90% estimated by HPLC. The solution of hydroxyapatite thus obtained was also analyzed by SDS-PAGE (Figure 7, well 5).

[0085] **Example 5:** Subsequent purification of G-CSF after chromatography on hydroxyapatite.

[0086] The example illustrates the subsequent stages of purifying G-CSF, which can be used after passing over hydroxyapatite in a multistage purification process.

[0087] From of a solution of hydroxyapatite of human GA-GCSF obtained according to the process of the invention, a stage of chromatography on a cation exchanger, then a stage of gel filtration chromatography were carried out in the following manner:

[0088] 1) chromatography on a cation exchanger.

[0089] A Pharmacia XK 26/40 column provided with 170 ml of SP SEPHAROSE® Fast Flow (Pharmacia) was equilibrated by washing at a flow rate of 13.2 ml/min with 1.380 litres of Milli-Q water, then with 1.380 litres of 20 mM sodium acetate buffer at pH 5.3 (20 mM buffer, pH 5.3).

[0090] 390 ml of hydroxyapatite solution of GA-GCSF obtained in Example 4 was applied to the column of SP SEPHAROSE® (Pharmacia) at a flow rate of 13.2 ml/min. The column was then washed at the same flow rate with 414 ml of 20 mM buffer, pH 5.3, then with 1 litre of elution buffer corresponding to a gradient of NaCl, varying from 0 to 250 mM in 5 column volumes (850 ml) of the 20 mM buffer, pH 5.3 over 52 minutes. The column effluent was collected in fractions of 13.2 ml. By detection of the total proteins in the column effluent by absorption at 280 nm, the elution of a protein peak is observed. The fractions collected were analyzed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA. The combined fractions (237 ml) contained 255 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 78.8% and a purity of 98.7%.

[0091] The solution of SP SEPHAROSE® (Pharmacia) thus obtained was also analyzed by SDS-PAGE (Figure 7, well 6).

[0092] 2) gel filtration chromatography.

[0093] 220 ml of the solution of SP SEPHAROSE® (Pharmacia) of GA-GCSF obtained above was concentrated approximately 10 times beforehand by ultrafiltration in a 300 ml Amicon cell provided with a PLGC membrane (Millipore), at + 4°C and under a nitrogen pressure of 2 bars. The UF concentrate thus obtained (21 ml) contained 253 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 107.1%. The UF concentrate was also analyzed by SDS-PAGE (Figure 7, well 7).

[0094] The UF concentrate was then subjected to a stage of gel filtration chromatography in the following manner:

[0095] Two Pharmacia XK 26/40 columns, each provided with 150 ml of Superdex™ 200 prep grade (Pharmacia) and equilibrated by washing with 1.35 litres of Milli-Q water at a flow rate of 3.3 ml/min, were set up in series, then equilibrated with 2.650 litres of PBS buffer (1X) at the same flow rate. 265 ml of Superdex™ 200 prep grade using two columns was thus obtained.

[0096] 10 ml of concentrated solution of SP SEPHAROSE® (Pharmacia) of GA-GCSF obtained above was applied to the sequence of columns at a flow rate of 3.3 ml/min. The columns were then washed at the same flow rate with 300 ml of PBS buffer (1X). By detection of the total proteins in the column effluent by absorption at 280 nm, the elution of a protein peak is observed. The collected fractions were analyzed for their G-CSF content by analytical RP-HPLC chromatography. The combined fractions (42.9 ml) contained 85.3 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 70.7% and a purity greater than 99%.

[0097] Similarly, 10 ml of the concentrated solution of SP SEPHAROSE® (Pharmacia) of GA-GCSF obtained above was chromatographed by gel filtration under the conditions indicated above, but by using a 20 mM sodium acetate buffer, pH 5.5, TWEEN® (ICI Americas, Delaware, USA) 20 (0.005%) in the place of the PBS buffer (1X). The combined fractions (42.9 ml) contained 95.4 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 79.1% and a purity greater than 99%.

[0098] Figure 7 shows the analysis by SDS-PAGE of the solution of filtration gel obtained in the PBS buffer (well 9) and in the acetate buffer, pH 5.5 (well 11) respectively.

[illegible]

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[illegible]

10) Process according to claim 1 in which the stage of passing over hydroxyapatite is carried out in a buffer of ionic strength comprised between 2 and 30 mSi and at a pH comprised between 5.5 and 7.5.

11) Process according to claim 10 in which the buffer comprises phosphate at a concentration comprised between 1 and 10 mM.

12) Process according to claim 10 in which the buffer is a 1 mM phosphate buffer and the pH is comprised between 6.0 and 7.5.

13) Process for purifying G-CSF which can be included in a multistage process for purifying G-CSF from a biological sample comprising the stages of

- a) reducing the volume of the biological sample containing the G-CSF by hydrophobic-interaction chromatography in order to obtain a concentrated, desalted and enriched fraction,
- b) passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound in order to obtain a concentrated, desalted and enriched fraction containing G-CSF and
- c) collecting the G-CSF.

14) Process according to claim 13 in which the multistage process moreover comprises one or more stages of chromatography chosen from the group constituted by ion exchange, reversed phase gel filtration, or affinity chromatography.

15) Process for eliminating the contaminating proteins from a solution containing G-CSF and the contaminating proteins comprising

- a) passing the solution over hydroxyapatite by which the contaminating proteins are fixed to the hydroxyapatite and the G-CSF is weakly bound and
- b) elution of the G-CSF.

16) Process according to claim 15 in which the elution of the G-CSF is carried out by simple washing with the fixation buffer.

17) Process according to claim 15 in which the solution containing the G-CSF is prepared by hydrophobic-interaction chromatography of a biological sample containing G-CSF.

Process for purifying granulocyte colony stimulating factor.

[0001] **BACKGROUND OF THE INVENTION AND RELEVANT ART**

[0002] The present invention relates to a process for purifying granulocyte colony stimulating factor (called G-CSF) by chromatography using a stage of chromatography on hydroxyapatite.

[0003] Among the colony stimulating factors which regulate the differentiation and the proliferation of mammalian hematopoietic cells, granulocyte colony stimulating factors have been described, for example in International Patent Application WO 87/01132 or European Patent Application EP169566.

[0004] The preparation of G-CSFs from different origins and their purification has been described in numerous scientific publications or patent applications. For example, European Patent Application EP 243153 described a process for purifying human G-CSF from cell lines of bladder carcinoma HBT5637; European Patent Application EP215126 described the purification of recombinant human G-CSF produced in *E. coli*. The processes described above correspond to multiple stages of purification in which the initial concentration of the starting biological preparations is generally obtained by standard methods of ultrafiltration or precipitation by a salt, followed by successive stages of reversed phase liquid chromatography (called RP-HPLC), which has the known drawback of leading to a significant loss of yield, for example because the protein is denatured by the organic solvents. Moreover, the American Patent US 5,055,555 described a selective and simplified process for purifying recombinant human G-CSF produced in a yeast on a larger scale, by precipitation by NaCl preceded by concentration by chromatography on a cation ion exchange column (S [Sepharose®] SEPHAROSE® or [Mono S®] MONO S® (Pharmacia)), but of which the yield and the purity obtained are not mentioned.

[0005] In addition, several uses of chromatography stages, other than RP-HPLC, have also been described for purifying G-CSFs:

[0006] The use of [Phenyl Sepharose] PHENYL SEPHAROSE® CL-6B (Pharmacia) was described by N A. Nicola et al., Journal of Biological Chemistry, Vol. 258, p. 9017-9023, 1983 for purifying G-CSF produced naturally by murine leukaemia cells. After preliminary

concentration of the medium on hollow fibre and "salting out" chromatography, the G-CSF was directly fixed on the [Phenyl Sepharose] PHENYL SEPHAROSE[®] (Pharmacia) column, then eluted using a decreasing salt gradient, then a linear gradient of ethylene glycol.

[0007] The use of hydroxyapatite was described by T. Arakawa et al., Archives of Biochemistry and Biophysics, Vol. 316, p. 285-289, 1995 as a last stage of purifying G-CSF produced from transformed CHO cells.

[0008] The use of SP [Sepharose] SEPHAROSE[®] Fast Flow (Pharmacia) was described by S-H Kang et al., Biotechnology Letters, Vol. 17, p. 687-692, 1995 for purifying G-CSF produced from transformed *E. coli* cells. After solubilization of the inclusion bodies and renaturation, the G-CSF was eluted using an NaCl gradient ranging from 0 to 0.5 M.

[0009]

DESCRIPTION OF THE INVENTION

[0010] One of the subjects of the present invention is to provide a process which allows the isolation and purification of G-CSFs, on a large scale and with high yields, by a stage of chromatography on hydroxyapatite from biological samples previously concentrated and enriched using hydrophobic-interaction chromatography.

[0011] The process of the invention can be used, for example, as a first stage of purifying G-CSF in a multistage process for the preparation of a G-CSF having a purity suitable for clinical use.

[0012] A subject of the invention is a process for purifying G-CSF from a biological sample comprising the stages of

- a) reducing the volume of the biological sample containing the G-CSF by hydrophobic-interaction chromatography in order to obtain a concentrated, desalted, and enriched fraction,
- b) passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound in order to obtain a concentrated, desalted, and enriched fraction containing G-CSF, and
- c) collecting the G-CSF.

[0013] The above process allows the G-CSF to be purified under non-denaturing conditions and to isolate the biologically active G-CSF.

[0017] In a quite unexpected and advantageous manner, the process according to the invention comprises a hydrophobic-interaction chromatography characterized by fixation of the protein at low conductivity or at a low salt content, for example ammonium sulphate or NaCl, and allows the volume of the initial biological sample to be reduced whilst eliminating the salts as well as a high percentage of the contaminating proteins. The process of the invention therefore allows a concentrated, desalted, and enriched in non-denatured G-CSF fraction to be obtained which is then passed over hydroxyapatite.

[0018] Contrary to the use of hydroxyapatite as a final stage of purification described by T. Arakawa et al., 1995 above, in which the G-CSF is collected in the unfixed fraction on the hydroxyapatite equilibrated in a 10 mM phosphate buffer containing 0.1 M NaCl at pH 7.0, the process according to the invention uses conditions where the G-CSF is weakly bound to the hydroxyapatite and thus allows a concentrated and desalted solution of purified G-CSF to be collected.

[0019] A particular subject of the invention is the above process in which the G-CSF collected has a purity of at least 90%.

[0020] A particular subject of the invention is also the above process in which the biological sample is a cell culture supernatant as well as the process in which the G-CSF is a human G-CSF (called hG-CSF).

[0021] A particular subject of the invention is also the above process in which the volume reduction stage comprises the [placing] placing of the biological sample on a hydrophobic-interaction chromatography support of phenyl type under conditions allowing the fixation of the G-CSF, then its elution.

[0022] A more particular subject of the invention is the above process in which the support of phenyl type is [Phenyl Sepharose] PHENYL SEPHAROSE[®] (Pharmacia).

[0023] A quite particular subject of the invention is the above process in which fixation on [Phenyl Sepharose] PHENYL SEPHAROSE[®] (Pharmacia) is carried out in a buffer with an ionic strength comprised between 0 and 60 mSi, and elution is carried out by reducing the ionic strength or the concentration of salt in the fixation buffer.

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[0025] A quite particular subject of the invention is the above process in which the fixation on [Phenyl Sepharose] PHENYL SEPHAROSE[®] (Pharmacia) is carried out in a buffer containing NaCl at a concentration comprised between 0.1 and 0.5 M, and elution is carried out [by] using water.

[0026] Examples of using hydrophobic-interaction chromatography on [Phenyl Sepharose] PHENYL SEPHAROSE[®] (Pharmacia) illustrating the process according to the invention are described further on in the experimental part.

[0027] A particular subject of the invention is also the above process according to the invention in which the stage of passing over hydroxyapatite is carried out in a buffer with an ionic strength comprised between 2 and 30 mSi and at a pH comprised between 5.5 and 7.5.

[0028] A more particular subject of the invention is the above process in which the buffer comprises phosphate at a concentration comprised between 1 and 10 mM.

[0029] A more particular subject of the invention is also the above process in which the buffer is a 1 mM phosphate buffer and the pH is comprised between 6.0 and 7.5.

[0030] Examples of using hydroxyapatite following chromatography on [Phenyl Sepharose] PHENYL SEPHAROSE[®] (Pharmacia) illustrating the process according to the invention are described further on in the experimental part.

[0031] The invention also relates to a process of purifying G-CSF which can be included in a multistage process for purifying G-CSF from a biological sample comprising the stages of

- a) reducing the volume of the biological sample containing the G-CSF by hydrophobic-interaction chromatography in order to obtain a concentrated, desalted, and enriched fraction,
- b) passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound in order to obtain a concentrated, desalted, and enriched fraction containing the G-CSF, and
- c) collecting the G-CSF.

[illegible]

[0033] Examples of stages of ion-exchange and gel filtration chromatography illustrating the use of the process according to the invention in a multistage process for purifying G-CSF are described further on in the experimental part.

[0034] The invention also relates to a process to eliminate the contaminating proteins from a solution containing G-CSF and contaminating proteins comprising:

- a) passing of the solution over hydroxyapatite by which the contaminating proteins are fixed to the hydroxyapatite and the G-CSF is weakly bound, and
- b) [elution of] eluting the G-CSF.

[0035] The invention particularly relates to the above process in which elution of the G-CSF is carried out by simple washing with the fixation buffer.

[0036] The contaminating proteins present in the solutions containing G-CSF have, for example, been added to the cell culture media. The added proteins can be, for example, serum, such as bovine serum or foetal calf serum, for example partially purified serum proteins, such as albumin or transferrin or mixtures of them.

[0037] The process according to the invention allows these contaminating proteins to be eliminated by passing the solution containing G-CSF over hydroxyapatite during the course of which the undesirable proteins are strongly fixed onto the medium and retained during elution of the G-CSF.

[0038] The invention also particularly relates to the above process in which the solution containing the G-CSF is prepared by hydrophobic-interaction chromatography of a biological sample containing G-CSF.

[0039] Preferably, the hydrophobic-interaction chromatography is carried out on a phenyl type support, for example on [Phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) as illustrated further on in the experimental part. The process according to the invention allows these contaminating proteins to be advantageously eliminated during the course of the first stage of purifying G-CSF from a biological sample.

[0040] **Analytical methods**

[0041] 1. Assay of G-CSF by HPLC

[0042] The fractions collected after chromatography were [analysed] analyzed by analytical RP-HPLC on a Vydac C4 column (0.46 x 15) , 300Å, 5 microns, equilibrated in H₂O/TFA 0.1%, at a flow rate of 2 ml/[mn] min with a linear gradient of acetonitrile/TFA 0.1% varying from 40 to 80 % over 10 minutes, and spectrophotometric detection at 214 nm.

[0043] The G-CSF [is] was eluted at a concentration of approximately 65% acetonitrile. The G-CSF concentration [is] was measured by comparison with a G-CSF standard. Evaluation of the purity [is] was measured by the ratio of the area of the G-CSF peak and the area of all peaks other than that of the injection peak.

[0044] 2. Assay of G-CSF by ELISA

[0045] The G-CSF concentration [is] was measured by using the ELISA kit from R&D System Inc. and the protocol recommended by the supplier.

[0046] 3. SDS-PAGE

[0047] The samples [are analysed] were analyzed on ready-to-use polyacrylamide gels (Novex) containing a gradient of 10 to 20 % polyacrylamide and a silver stain using the Biorad silver staining kit for a deposit of 50 ng to 1 µg of G-CSF.

[0048] **BRIEF DESCRIPTION OF THE FIGURES**

[0049] The attached figures illustrate certain aspects of the invention.

[0050] Figure 1 is a chromatogram showing the fractionation on [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) of a supernatant of cells expressing GA-GCSF and containing 0.1 M NaCl. The arbitrary units represent the conductivity and the optical density (OD) respectively of the column effluent expressed as a percentage.

[0051] Figure 2 is a chromatogram showing the fractionation on [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) of a supernatant of cells expressing GA-GCSF and containing 0.5 M NaCl. The arbitrary units have the same meaning as in Figure 1.

[0052] Figure 3 is a chromatogram showing the fractionation of GA-GCSF on hydroxyapatite [MacroPrep] MACROPREP® (BioRad) ceramic Type I after [phenyl

Sepharose] PHENYL SEPHAROSE® (Pharmacia). The arbitrary units have the same meaning as in Figure 1.

[0053] Figure 4 is a chromatogram of analytical RP-HPLC of GA-GCSF after [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) and hydroxyapatite Type I.

[0054] Figure 5 is a chromatogram showing the fractionation of GA-GCSF on hydroxyapatite [MacroPrep] MACROPREP® (BioRad) ceramic Type II after [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia). The arbitrary units have the same meaning as in Figure 1.

[0055] Figure 6 is a chromatogram of analytical RP-HPLC of GA-GCSF after [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) and hydroxyapatite Type II.

[0056] Figure 7 represents analysis by SDS-PAGE of the successive purification of GA-GCSF in a supernatant of filtered culture (well 3), an eluate of [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) (well 4), an eluate of hydroxyapatite (well 5), an eluate of [SP Sepharose] SP SEPHAROSE® (Pharmacia) (well 6), a UF concentrate (well 7), an eluate of filtration gel in PBS buffer (well 9), and an eluate of filtration gel in acetate buffer pH 5.5 (well 11), with standard molecular weight markers (well 1). The band corresponding to the apparent MW of the GA-GCSF is indicated by an arrow.

[0057] EXAMPLES

[0058] **Example 1:** Concentration of a biological sample of G-CSF by chromatography on [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia).

[0059] The starting material [is] was the centrifugation supernatant of a culture broth of human cell lines expressing a human GA-GCSF obtained according to the International Patent Application WO95/31560 in [a Endotronics] an ENDOTRONICS® (Minneapolis, MN) hollow fibre bioreactor in DMEM/F12 (Hyclone) medium containing 0.9% foetal calf serum. After centrifugation, the supernatant was stored at -20°C before use.

[0060] The thawed supernatant was chromatographed on [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) after adding 0.1 M NaCl q.s. and filtration on a 0.22 µm Millipore membrane, at a temperature of approximately 15 to 20°C.

[0061] Using a Pharmacia XK16 column (1.6 cm x 40 cm) provided with 50 ml of [Phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) Fast Flow High Substitution (Pharmacia), stored under ethanol at 25%, then washed with Milli-Q [demineralised] de-mineralized water before use, then equilibrated with a 0.1M solution of NaCl, concentration by chromatography on [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) was carried out in the following manner: 2295 ml of salted and filtered supernatant obtained above (conductivity 17.7 mS.cm^{-1}) [is] was applied to the column at a flow rate of 13 ml/[mn] min, and [collecting] the effluent of the column was collected in fractions of 500 ml. The column [is] was then washed at a flow rate of 4 ml/[mn] min with 220 ml of a 0.05 M NaCl solution, and [collecting] the effluent of the column was collected in fractions of 40 ml. The column [is] was then eluted at the same flow rate with 150 ml of Milli-Q demineralized water, and [collecting] the effluent from the column was collected in fractions of 2 ml. The column [is] was finally regenerated by washing at the same flow rate with an 8 M solution of urea.

[0062] The total proteins in the effluent of the column [are] were detected by absorption at 280 nm and the salt concentration [is] was monitored using a conductimeter. The presence in the column effluent of a first peak of protein eluted by water, then of a second peak of protein eluted by washing with urea is shown in Figure 1.

[0063] The fractions collected during elution with water were [analysed] analyzed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA using the conditions described previously. The fractions containing the combined G-CSF (40 ml) [contain] contained 29.3 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 56% and a purity of 58%.

[0064] The solution of GA-GCSF [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) thus obtained [has] had a conductivity of 0.161 mS.cm^{-1} .

[0065] **Example 2:** Chromatography on [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia), then on hydroxyapatite as a first stage for purifying G-CSF.

[0066] The starting material [is] was the supernatant of a culture broth of human cell lines expressing a human GA-GCSF obtained as in Example 1 but using a 5 litre bioreactor in the place of an [Endotronics] ENDOTRONICS® (Minneapolis, MN) bioreactor. The thawed supernatant was chromatographed on [phenyl Sepharose] PHENYL SEPHAROSE®

(Pharmacia) after the addition of 0.5 M NaCl q.s. and filtration on a 0.45 μ m Millipore membrane.

[0067] By using a Pharmacia XK16 column provided with 50 ml of [Phenyl Sepharose®] PHENYL SEPHAROSE® (Pharmacia) Fast Flow High Substitution (Pharmacia) stored under ethanol at 25%, then washing with Milli-Q demineralized water before use, concentration by chromatography on [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) was carried out in the following manner: 1640 ml of salted and filtered supernatant obtained above (conductivity 56.3 mS.cm⁻¹) [is] was applied to the column at the flow rate of 4 ml/[mn] min and the effluent of the column [is] was collected in fractions of 400 ml. The column [is] was then eluted at the same flow rate of 4 ml/[mn] min with 240 ml of 0.5M NaCl, and [collecting] the effluent of the column was collected in fractions of 40 ml. The column [is] was then eluted at the same flow rate with 150 ml of Milli-Q [demineralised] de-mineralized water, and the [collecting] the effluent of the column was collected in fractions of 2 ml. The column [is] was finally regenerated by washing with the same flow rate with a solution of 8M urea.

[0068]. The total proteins in the column effluent and the salt concentration [are] were detected as in Example 1. The presence in the column effluent of a first peak of the protein eluted by washing with water, then of a second peak of the protein eluted by washing with urea is shown in Figure 2.

[0069] The fractions collected during elution with water were [analysed] analyzed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA. The combined fractions containing the combined G-CSF (50 ml) [contain] contained 45.1 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 90% with a purity of 61%.

[0070] The solution of GA-GCSF [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) thus obtained [has] had a conductivity of 4.22 mS.cm⁻¹, which [allows] allowed it to be used as it is in the following stage of chromatography on hydroxyapatite.

[0071] Using a Pharmacia XK16 column provided with 29 g of hydroxyapatite [Macro-Prep] MACROPREP® Ceramic, Type I (Bio-Rad), previously suspended in 250 mM sodium phosphate buffer at pH 7.3 (250 mM buffer, pH 7.3), then equilibrated by percolation with a flow rate of 5 ml/[mn] min of 500 ml of the 250 mM buffer diluted to 1/250 (1 mM buffer,

[0077] **Example 4:** Chromatography on [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia), then on hydroxyapatite as a first stage of large scale purification of G-CSF.

[0078] The starting material [is] was a supernatant of a culture broth of human cell lines expressing GA-GCSF obtained according to Example 2 but using a 100 litre bioreactor and the medium without calf serum.

[0079] 10.5 litres of supernatant, previously concentrated by ultrafiltration then stored at -20°C before use and corresponding to 84 litres of starting broth, was chromatographed on [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) after adding 0.307 kg of NaCl (q.s. 0.5 M), then [filtration] filtered on Durieux N°127 paper. Analysis by SDS-PAGE of the supernatant of filtered culture thus obtained is shown in Figure 7. (well 3).

[0080] By using a Pharmacia XK 50/30 column provided with 500 ml of [Phenyl Sepharose®] PHENYL SEPHAROSE® (Pharmacia) Fast Flow High Substitution (Pharmacia) stored under ethanol at 25%, then equilibrated with a solution of 0.5 M NaCl before use, concentration by chromatography on [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) was carried out in the following manner: the concentrated salted supernatant obtained above (conductivity 40 mS.cm⁻¹) [is] was applied to the column at a flow rate of 40 ml/[mn] min. The column [is] was then successively eluted at the same flow rate with 1.5 litres of 0.5 M NaCl, then with 1.5 litres of Milli-Q water, and [collecting] the column effluent was collected in fractions of 20 ml. The total proteins and the concentration of salts [are] were detected as in Example 1.

[0081] The fractions corresponding to the peak of the protein eluted with water were [analysed] analyzed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA. The combined fractions containing the G-CSF (400 ml) [contain] contained 357 mg of GA-CSF titrated by HPLC, corresponding to a yield of 67.9% with a purity of 20.6%. The solution of GA-GCSF [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) thus obtained [has] had a conductivity of 2 mS.cm⁻¹ at 20°C.

[0082] The solution of [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) was also analyzed by SDS-PAGE (Figure 7, well 4). After stabilization by adding Pefabloc (0.2

mg/ml) and benzamidine (1mM), the solution was immediately used for the following stage of chromatography on hydroxyapatite.

[0083] By using a Pharmacia XK 50/30 column provided with 290 g of hydroxyapatite [Macro-Prep®] MACROPREP® Ceramic, Type II (Bio-Rad) previously suspended in 5 litres of 1 mM sodium phosphate buffer at pH 6 (1 mM buffer, pH 6), then equilibrated by percolation of 1 litre of 250 mM sodium phosphate buffer at pH 6 (250 mM buffer, pH 6) at a flow rate of 50 ml/[mn] min, then by percolation of 5 litres of 1 mM buffer, pH 6 (thus generating a column of 500 ml of hydroxyapatite), chromatography on hydroxyapatite was carried out in the following manner: 400 ml of the solution of CA-GCSF of [stabilised] stabilized [phenyl Sepharose] PHENYL SEPHAROSE® (Pharmacia) obtained above [is] was applied to the column of hydroxyapatite at a flow rate of 50 ml/[mn] min. The column [is] was then eluted with 1.50 litres of 1 mM buffer, pH 6, collecting the column effluent in fractions of 50 ml. The total proteins as well as the conductivity of the column effluent [are] were detected as indicated in Example 1.

[0084] The fractions collected were [analysed] analyzed by analytical RP-HPLC and by ELISA. The combined fractions (400 ml) [contain] contained 331 mg of GA-GCSF titrated by HPLC, corresponding to a chromatography yield of 92.5% with a purity higher than the 90% estimated by HPLC. The solution of hydroxyapatite thus obtained was also [analysed] analyzed by SDS-PAGE (Figure 7, well 5).

[0085] **Example 5:** Subsequent purification of G-CSF after chromatography on hydroxyapatite.

[0086] The example illustrates the subsequent stages of purifying G-CSF, which can be used after passing over hydroxyapatite in a multistage purification process.

[0087] From of a solution of hydroxyapatite of human GA-GCSF obtained according to the process of the invention, a stage of chromatography on a cation exchanger, then a stage of gel filtration chromatography were carried out in the following manner:

[0088] 1) chromatography on a cation exchanger.

[0089] A Pharmacia XK 26/40 column provided with 170 ml of SP [Sepharose] SEPHAROSE® Fast Flow (Pharmacia) was equilibrated by washing at a flow rate of 13.2

ml/[mn] min with 1.380 litres of Milli-Q water, then with 1.380 litres of 20 mM sodium acetate buffer at pH 5.3 (20 mM buffer, pH 5.3).

[0090] 390 ml of hydroxyapatite solution of GA-GCSF obtained in Example 4 [is] was applied to the column of SP [Sephacrose] SEPHAROSE® (Pharmacia) at a flow rate of 13.2 ml/[mn] min. The column [is] was then washed at the same flow rate with 414 ml of 20 mM buffer, pH 5.3, then with 1 litre of elution buffer corresponding to a gradient of NaCl, varying from 0 to 250 mM in 5 column volumes (850 ml) of the 20 mM buffer, pH 5.3 over 52 minutes. The, collecting the] column effluent was collected in fractions of 13.2 ml. By detection of the total proteins in the column effluent by absorption at 280 nm, the elution of a protein peak is observed. The fractions collected were [analysed] analyzed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA. The combined fractions (237 ml) [contain] contained 255 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 78.8% and a purity of 98.7%.

[0091] The solution of SP [Sephacrose] SEPHAROSE® (Pharmacia) thus obtained was also [analysed] analyzed by SDS-PAGE (Figure 7, well 6).

[0092] 2) gel filtration chromatography.

[0093] 220 ml of the solution of SP [Sephacrose] SEPHAROSE® (Pharmacia) of GA-GCSF obtained above was concentrated approximately 10 times beforehand by ultrafiltration in a 300 ml Amicon cell provided with a PLGC membrane (Millipore), at + 4°C and under a nitrogen pressure of 2 bars. The UF concentrate thus obtained (21 ml) [contains] contained 253 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 107.1%. The UF concentrate was also [analysed] analyzed by SDS-PAGE (Figure 7, well 7).

[0094] The UF concentrate was then subjected to a stage of gel filtration chromatography in the following manner:

[0095] Two Pharmacia XK 26/40 columns, each provided with 150 ml of Superdex™ 200 prep grade (Pharmacia) and equilibrated by washing with 1.35 litres of Milli-Q water at a flow rate of 3.3 ml/[mn] min, were set up in series, then equilibrated with 2.650 litres of PBS buffer (1X) at the same flow rate. 265 ml of Superdex™ 200 prep grade using two columns [is] was thus obtained.

[0096] 10 ml of concentrated solution of SP [Sephacrose] SEPHAROSE® (Pharmacia) of GA-GCSF obtained above [is] was applied to the sequence of columns at a flow rate of 3.3 ml/[mn] min. The columns [are] were then washed at the same flow rate with 300 ml of PBS buffer (1X). By detection of the total proteins in the column effluent by absorption at 280 nm, the elution of a protein peak is observed. The collected fractions were [analysed] analyzed for their G-CSF content by analytical RP-HPLC chromatography. The combined fractions (42.9 ml) [contain] contained 85.3 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 70.7% and a purity greater than 99%.

[0097] Similarly, 10 ml of the concentrated solution of SP [Sephacrose] SEPHAROSE® (Pharmacia) of GA-GCSF obtained above [is] was chromatographed by gel filtration under the conditions indicated above, but by using a 20 mM sodium acetate buffer, pH 5.5, [Tween] TWEEN® (ICI Americas, Delaware, USA) 20 (0.005%) in the place of the PBS buffer (1X). The combined fractions (42.9 ml) [contain] contained 95.4 mg of GA-GCSF titrated by HPLC, corresponding to a yield of 79.1% and a purity greater than 99%.

[0098] Figure 7 shows the analysis by SDS-PAGE of the solution of filtration gel obtained in the PBS buffer (well 9) and in the acetate buffer, pH 5.5 (well 11) respectively.

$$\frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} f(x) e^{-x^2} dx = \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} f(x) e^{-x^2} dx$$

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9) Process according to claim 8 in which the fixation on Phenyl Sepharose[®] is carried out in a buffer containing NaCl at a concentration comprised between 0.1 and 0.5 M and the elution is carried out with water.

10) Process according to claim 1 in which the stage of passing over hydroxyapatite is carried out in a buffer of ionic strength comprised between 2 and 30 mSi and at a pH comprised between 5.5 and 7.5.

11) Process according to claim 10 in which the buffer comprises phosphate at a concentration comprised between 1 and 10 mM.

12) Process according to claim 10 in which the buffer is a 1 mM phosphate buffer and the pH is comprised between 6.0 and 7.5.

13) Process for purifying G-CSF which can be included in a multistage process for purifying G-CSF from a biological sample comprising the stages of

- a) reducing the volume of the biological sample containing the G-CSF by hydrophobic-interaction chromatography in order to obtain a concentrated, desalted and enriched fraction,
- b) passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound in order to obtain a concentrated, desalted and enriched fraction containing G-CSF and
- c) collecting the G-CSF.

14) Process according to claim 13 in which the multistage process moreover comprises one or more stages of chromatography chosen from the group constituted by ion exchange, reversed phase gel filtration, or affinity chromatography.

15) Process for eliminating the contaminating proteins from a solution containing G-CSF and the contaminating proteins comprising

- a) passing the solution over hydroxyapatite by which the contaminating proteins are fixed to the hydroxyapatite and the G-CSF is weakly bound and
- b) elution of the G-CSF.

16) Process according to claim 15 in which the elution of the G-CSF is carried out by simple washing with the fixation buffer.

17) Process according to claim 15 in which the solution containing the G-CSF is prepared by hydrophobic-interaction chromatography of a biological sample containing G-CSF.

7/p.1>
Process for purifying granulocyte colony stimulating factor.

The present invention relates to a process for purifying granulocyte colony stimulating factor (called G-CSF) by chromatography using a stage of chromatography on hydroxyapatite.

Among the colony stimulating factors which regulate the differentiation and the proliferation of mammalian hematopoietic cells, granulocyte colony stimulating factors have been described for example in International Patent Application WO 87/01132 or European Patent Application EP169566.

The preparation of G-CSFs from different origins and their purification has been described in numerous scientific publications or patent applications. For example, European Patent Application EP 243153 described a process for purifying human G-CSF from cell lines of bladder carcinoma HBT5637; European Patent Application EP215126 described the purification of recombinant human G-CSF produced in *E. coli*. The processes described above correspond to multiple stages of purification in which the initial concentration of the starting biological preparations is generally obtained by standard methods of ultrafiltration or precipitation by a salt, followed by successive stages of reversed phase liquid chromatography (called RP-HPLC) which has the known drawback of leading to a significant loss of yield, for example because the protein is denatured by the organic solvents. Moreover, the American Patent US 5,055,555 described a selective and simplified process for purifying recombinant human G-CSF produced in a yeast on a larger scale, by precipitation by NaCl preceded by concentration by chromatography on a cation ion exchange column (S Sepharose® or Mono S®), but of which the yield and the purity obtained are not mentioned.

In addition, several uses of chromatography stages, other than RP-HPLC, have also been described for purifying G-CSFs:

The use of Phenyl Sepharose® CL-6B (Pharmacia) was described by N A. Nicola et al., Journal of Biological

Chemistry, Vol. 258, p. 9017-9023, 1983 for purifying G-CSF produced naturally by murine leukaemia cells. After preliminary concentration of the medium on hollow fibre and "salting out" chromatography, the G-CSF was directly fixed on the Phenyl Sepharose® column, then eluted using a decreasing salt gradient, then a linear gradient of ethylene glycol.

The use of hydroxyapatite was described by T. Arakawa et al., Archives of Biochemistry and Biophysics, Vol. 316, p. 285-289, 1995 as a last stage of purifying G-CSF produced from transformed CHO cells.

The use of SP Sepharose® Fast Flow (Pharmacia) was described by S-H Kang et al., Biotechnology Letters, Vol. 17, p. 687-692, 1995 for purifying G-CSF produced from transformed *E. coli* cells. After solubilization of the inclusion bodies and renaturation, the G-CSF was eluted using an NaCl gradient ranging from 0 to 0.5 M.

One of the subjects of the present invention is to provide a process which allows the isolation and purification of G-CSFs, on a large scale and with high yields, by a stage of chromatography on hydroxyapatite from biological samples previously concentrated and enriched using hydrophobic-interaction chromatography.

The process of the invention can be used for example as a first stage of purifying G-CSF in a multistage process for the preparation of a G-CSF having a purity suitable for clinical use.

A subject of the invention is a process for purifying G-CSF from a biological sample comprising the stages of

- reducing the volume of the biological sample containing the G-CSF by hydrophobic-interaction chromatography in order to obtain a concentrated, desalted and enriched fraction,
- passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound in order to obtain a concentrated, desalted and enriched fraction containing G-CSF and
- collecting the G-CSF.

The above process allows the G-CSF to be purified under non-denaturing conditions and to isolate the biologically

active G-CSF.

The purified G-CSF according to the process of the invention can be any known G-CSF of biological and pharmaceutical interest. By G-CSF, is included a G-CSF produced constitutively by cells, for example by cell lines established from tumourous cells as described by Watson et al., J. Immunol., Vol. 137, p. 854-857, 1986, a G-CSF produced by activation of the G-CSF gene (called GA-GCSF for "Gene Activation-GCSF") in human cells as described in International Patent Application WO 95/31560 or a G-CSF produced by recombinant DNA technology by host cells. The host cells can be eucaryotic cells such as mammalian cells, for example monkey COS cells, hamster CHO cells or mouse C127 cells or such as yeasts, for example *S. cerevisiae* or procaryotic cells, for example *E. coli*. Examples of recombinant G-CSFs have been described, for example in the European Patent Application EP217404 which described a G-CSF produced in C127 cells or in CHO cells, in the US Patent 5,055,555 which describes a G-CSF produced by *S. cerevisiae* or in the International Patent Application WO 87/01132 which describes a G-CSF produced in COS cells as well as a G-CSF produced in *E. coli*. The process allows both glycosylated or non-glycosylated G-CSFs to be purified.

The biological sample from which the process of the invention allows a G-CSF to be purified comprises the biological fluids of cell cultures such as cell lysates, inclusion bodies or culture supernatants when the G-CSF is excreted. The biological sample used for purifying the G-CSF was preferably separated beforehand from cells or from cell debris by methods known to a person skilled in the art, for example by filtration, by centrifugation or by ultrafiltration.

By hydrophobic-interaction chromatography, is meant a chromatography on a substance separation support on the basis of their differences of interaction with hydrophobic groups attached to a matrix without ionic groups. The hydrophobic group can be an aliphatic ligand, for example a butyl or octyl group or an aromatic ligand, for example a phenyl group

or a phenylbutylamine group and the matrix is generally a gel, for example agarose such as Sepharose®. The supports used are commercially available products. In all the processes of using hydrophobic-interaction chromatography, the fixation of proteins to hydrophobic gel is carried out in the presence of high concentrations of salts. In a quite unexpected and advantageous manner, the process according to the invention comprises a hydrophobic-interaction chromatography characterized by fixation of the protein at low conductivity or at a low salt content, for example ammonium sulphate or NaCl, and allows the volume of the initial biological sample to be reduced whilst eliminating the salts as well as a high percentage of the contaminating proteins. The process of the invention therefore allows a concentrated, desalted and enriched in non-denatured G-CSF fraction to be obtained which is then passed over hydroxyapatite.

Contrary to the use of hydroxyapatite as a final stage of purification described by T. Arakawa et al., 1995 above, in which the G-CSF is collected in the unfixed fraction on the hydroxyapatite equilibrated in a 10 mM phosphate buffer containing 0.1 M NaCl at pH 7.0, the process according to the invention uses conditions where the G-CSF is weakly bound to the hydroxyapatite and thus allows a concentrated and desalted solution of purified G-CSF to be collected.

A particular subject of the invention is the above process in which the G-CSF collected has a purity of at least 90 %.

A particular subject of the invention is also the above process in which the biological sample is a cell culture supernatant as well as the process in which the G-CSF is a human G-CSF (called hG-CSF).

A particular subject of the invention is also the above process in which the volume reduction stage comprises the placing of the biological sample on a hydrophobic-interaction chromatography support of phenyl type under conditions allowing the fixation of the G-CSF, then its elution.

A more particular subject of the invention is the above process in which the support of phenyl type is Phenyl Sepharose®.

5 A quite particular subject of the invention is the above process in which fixation on Phenyl Sepharose® is carried out in a buffer with an ionic strength comprised between 0 and 60 mSi and elution is carried out by reducing the ionic strength or the concentration of salt in the fixation buffer.

10 A more particular subject of the invention is also the above process in which fixation on Phenyl Sepharose® is carried out in a buffer containing NaCl at a concentration comprised between 0.1 and 1 M.

15 A quite particular subject of the invention is the above process in which the fixation on Phenyl Sepharose® is carried out in a buffer containing NaCl at a concentration comprised between 0.1 and 0.5 M and elution is carried out by water.

20 Examples of using hydrophobic-interaction chromatography on Phenyl Sepharose® illustrating the process according to the invention are described further on in the experimental part.

A particular subject of the invention is also the above process according to the invention in which the stage of passing over hydroxyapatite is carried out in a buffer with an ionic strength comprised between 2 and 30 mSi and at a pH
25 comprised between 5.5 and 7.5.

A more particular subject of the invention is the above process in which the buffer comprises phosphate at a concentration comprised between 1 and 10 mM.

30 A more particular subject of the invention is also the above process in which the buffer is a 1 mM phosphate buffer and the pH is comprised between 6.0 and 7.5.

35 Examples of using hydroxyapatite following chromatography on Phenyl Sepharose® illustrating the process according to the invention are described further on in the experimental part.

The invention also relates to a process of purifying G-CSF which can be included in a multistage process for purifying G-CSF from a biological sample comprising the

stages of

- a) reducing the volume of the biological sample containing the G-CSF by hydrophobic-interaction chromatography in order to obtain a concentrated, desalted and enriched fraction,
- 5 b) passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound in order to obtain a concentrated, desalted and enriched fraction containing the G-CSF and
- c) collecting the G-CSF.

10 The invention particularly relates to the above process in which the multistage process moreover comprises one or more stages of chromatography chosen from the group constituted by ion exchange, filtration gel, reverse phase or affinity chromatography.

15 Examples of stages of ion exchange and gel filtration chromatography illustrating the use of the process according to the invention in a multistage process for purifying G-CSF are described further on in the experimental part.

The invention also relates to a process to eliminate the
20 contaminating proteins from a solution containing G-CSF and contaminating proteins comprising:

- a) passing of the solution over hydroxyapatite by which the contaminating proteins are fixed to the hydroxyapatite and the G-CSF is weakly bound and
- 25 b) elution of the G-CSF.

The invention particularly relates to the above process in which elution of the G-CSF is carried out by simple washing with the fixation buffer.

The contaminating proteins present in the solutions
30 containing G-CSF have for example been added to the cell culture media. The added proteins can be for example serum, such as bovine serum or foetal calf serum, for example partially purified serum proteins, such as albumin or transferrin or mixtures of them.

35 The process according to the invention allows these contaminating proteins to be eliminated by passing the solution containing G-CSF over hydroxyapatite during the course of which the undesirable proteins are strongly fixed

onto the medium and retained during elution of the G-CSF.

The invention also particularly relates to the above process in which the solution containing the G-CSF is prepared by hydrophobic-interaction chromatography of a biological sample containing G-CSF.

Preferably, the hydrophobic-interaction chromatography is carried out on a phenyl type support, for example on Phenyl Sepharose® as illustrated further on in the experimental part. The process according to the invention allows these contaminating proteins to be advantageously eliminated during the course of the first stage of purifying G-CSF from a biological sample.

Analytical methods

1. Assay of G-CSF by HPLC

The fractions collected after chromatography were analysed by analytical RP-HPLC on a Vydac C4 column (0.46 x 15), 300Å, 5 microns, equilibrated in H₂O/TFA 0.1 %, at a flow rate of 2 ml/min with a linear gradient of acetonitrile/TFA 0.1 % varying from 40 to 80 % over 10 minutes, and spectrophotometric detection at 214 nm.

The G-CSF is eluted at a concentration of approximately 65 % acetonitrile. The G-CSF concentration is measured by comparison with a G-CSF standard. Evaluation of the purity is measured by the ratio of the area of the G-CSF peak and the area of all peaks other than that of the injection peak.

2. Assay of G-CSF by ELISA

The G-CSF concentration is measured by using the ELISA kit from R&D System Inc. and the protocol recommended by the supplier.

3. SDS-PAGE

The samples are analysed on ready-to-use polyacrylamide gels (Novex) containing a gradient of 10 to 20 % polyacrylamide and a silver stain using the Biorad silver staining kit for a deposit of 50 ng to 1µg of G-CSF.

The attached figures illustrate certain aspects of the invention.

Figure 1 is a chromatogram showing the fractionation on phenyl Sepharose of a supernatant of cells expressing GA-GCSF

and containing 0.1 M NaCl. The arbitrary units represent the conductivity and the optical density (OD) respectively of the column effluent expressed as a percentage.

5 Figure 2 is a chromatogram showing the fractionation on phenyl Sepharose of a supernatant of cells expressing GA-GCSF and containing 0.5 M NaCl. The arbitrary units have the same meaning as in Figure 1.

10 Figure 3 is a chromatogram showing the fractionation of GA-GCSF on hydroxyapatite MacroPrep® ceramic Type I after phenyl Sepharose. The arbitrary units have the same meaning as in Figure 1.

Figure 4 is a chromatogram of analytical RP-HPLC of GA-GCSF after phenyl Sepharose and hydroxyapatite Type I.

15 Figure 5 is a chromatogram showing the fractionation of GA-GCSF on hydroxyapatite MacroPrep® ceramic Type II after phenyl Sepharose. The arbitrary units have the same meaning as in Figure 1.

Figure 6 is a chromatogram of analytical RP-HPLC of GA-GCSF after phenyl Sepharose and hydroxyapatite Type II.

20 Figure 7 represents analysis by SDS-PAGE of the successive purification of GA-GCSF in a supernatant of filtered culture (well 3), an eluate of phenyl Sepharose (well 4), an eluate of hydroxyapatite (well 5), an eluate of SP Sepharose (well 6), a UF concentrate (well 7), an eluate of filtration gel in PBS buffer (well 9), an eluate of filtration gel in acetate buffer pH 5.5 (well 11) with standard molecular weight markers (well 1). The band corresponding to the apparent MW of the GA-GCSF is indicated by an arrow.

30 **Example 1:** Concentration of a biological sample of G-CSF by chromatography on phenyl Sepharose.

The starting material is the centrifugation supernatant of a culture broth of human cell lines expressing a human GA-GCSF obtained according to the International Patent Application WO95/31560 in a Endotronics® hollow fibre bioreactor in DMEM/F12 (Hyclone) medium containing 0.9 % foetal calf serum. After centrifugation, the supernatant was stored at -20°C before use.

Using a Pharmacia XK16 column (1.6 cm x 40 cm) provided with 50 ml of Phenyl Sepharose® Fast Flow High Substitution (Pharmacia), stored under ethanol at 25 %, then washed with Milli-Q demineralised water before use, then equilibrated with a 0.1M solution of NaCl, concentration by chromatography on phenyl Sepharose was carried out in the following manner: 2295 ml of salted and filtered supernatant obtained above (conductivity 17.7 mS.cm⁻¹) is applied to the column at a flow rate of 13 ml/mn collecting the effluent of the column in fractions of 500 ml. The column is then washed at a flow rate of 4 ml/mn with 220 ml of 0.05 M NaCl solution collecting the effluent of the column in fractions of 40 ml. The column is then eluted at the same flow rate with 150 ml of Milli-Q demineralized water collecting the effluent from the column in fractions of 2 ml. The column is finally regenerated by washing at the same flow rate with an 8 M solution of urea.

The total proteins in the effluent of the column are detected by absorption at 280 nm and the salt concentration is monitored using a conductimeter. The presence in the column effluent of a first peak of protein eluted by water, then of a second peak of protein eluted by washing with urea is shown in Figure 1.

The fractions collected during elution with water were analysed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA using the conditions described previously. The fractions containing the combined G-CSF (40 ml) contain 29.3 mg of GA-GCSF titrated by HPLC corresponding to a yield of 56 % and a purity of 58 %.

The solution of GA-GCSF phenyl Sepharose thus obtained
35 has a conductivity of 0.161 mS.cm^{-1} .

Example 2: Chromatography on phenyl Sepharose, then on hydroxyapatite as a first stage for purifying G-CSF.

The starting material is the supernatant of a culture

broth of human cell lines expressing a human GA-GCSF obtained as in Example 1 but using a 5 litre bioreactor in the place of an Endotronics® bioreactor. The thawed supernatant was chromatographed on phenyl Sepharose after the addition of 0.5 M NaCl q.s. and filtration on a 0.45 µm Millipore membrane.

By using a Pharmacia XK16 column provided with 50 ml of Phenyl Sepharose® Fast Flow High Substitution (Pharmacia) stored under ethanol at 25 %, then washing with Milli-Q demineralized water before use, concentration by chromatography on phenyl Sepharose was carried out in the following manner:
1640 ml of salted and filtered supernatant obtained above (conductivity 56.3 mS.cm⁻¹) is applied to the column at the flow rate of 4 ml/mn and the effluent of the column is collected in fractions of 400 ml. The column is then eluted at the same flow rate of 4 ml/mn with 240 ml of 0.5M NaCl collecting the effluent of the column in fractions of 40 ml. The column is then eluted at the same flow rate with 150 ml of Milli-Q demineralised water collecting the effluent of the column in fractions of 2 ml. The column is finally regenerated by washing with the same flow rate with a solution of 8M urea.

The total proteins in the column effluent and the salt concentration are detected as in Example 1. The presence in the column effluent of a first peak of the protein eluted by washing with water, then of a second peak of the protein eluted by washing with urea is shown in Figure 2.

The fractions collected during elution with water were analysed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA. The combined fractions containing the combined G-CSF (50 ml) contain 45.1 mg of GA-GCSF titrated by HPLC corresponding to a yield of 90 % with a purity of 61 %.

The solution of GA-GCSF phenyl Sepharose thus obtained has a conductivity of 4.22 mS.cm⁻¹ which allows it to be used as it is in the following stage of chromatography on hydroxyapatite.

Using a Pharmacia XK16 column provided with 29 g of

hydroxyapatite Macro-Prep® Ceramic, Type I (Bio-rad), previously suspended in 250 mM sodium phosphate buffer at pH 7.3 (250 mM buffer, pH 7.3), then equilibrated by percolation with a flow rate of 5 ml/min of 500 ml of the 250 mM buffer
 5 diluted to 1/250 (1 mM buffer, pH 7.3), chromatography on hydroxyapatite was carried out in the following manner: 24 ml of the solution of GA-GCSF phenyl Sepharose obtained above, then stored overnight at +2°C, is applied to the hydroxyapatite column at the same flow rate. The column is
 10 then eluted with 150 ml of 1 mM buffer, pH 7.3, then regenerated by washing with 250 mM buffer, pH 7.3 at the same flow rate collecting the column effluent in fractions of 5ml. The total proteins in the column effluent and the salt concentration are detected as in Example 2.

15 The presence in the column effluent of a peak of protein eluted with the 1 mM buffer, pH 7.3, then of a second peak of protein eluted with the 250 mM buffer, pH 7.3 is shown in Figure 3.

The fractions collected during elution with the 1 mM
 20 buffer, pH 7.3 were analyzed by analytical RP-HPLC and by ELISA. The combined fractions 25 to 49 (25 ml) contain 21.3 mg of GA-GCSF titrated by HPLC corresponding to a yield of 98.4 % with a purity of 97.8 % and gives a homogenous peak in HPLC (Figure 4).

25 **Example 3:** Chromatography on phenyl Sepharose, then on hydroxyapatite as a first stage of purifying G-CSF. 24 ml of the solution of GA-GCSF phenyl Sepharose obtained in Example 2 was chromatographed on a column of hydroxyapatite according to the conditions described in Example 2, but using
 30 the hydroxyapatite Macro-Prep® Ceramic Type II (Biorad) instead of Type I.

The presence in the column effluent of a first peak of protein eluted by the 1 mM buffer, pH 7.3, then of a second peak of protein eluted by the 250 mM buffer, pH 7.3 is shown
 35 in Figure 5. The fractions collected during the elution with 1 mM buffer, pH 7.3 were analyzed by analytical RP-HPLC and by ELISA. The combined fractions (25 ml) contain 21.7 mg of GA-GCSF titrated by HPLC corresponding to a yield of 100.2 %

with a purity of 94.5 % and gives a homogenous peak in HPLC (Figure 6).

Example 4: Chromatography on phenyl Sepharose, then on hydroxyapatite as a first stage of large scale purification of G-CSF.

The starting material is a supernatant of a culture broth of human cell lines expressing GA-GCSF obtained according to Example 2 but using a 100 litre bioreactor and the medium without calf serum.

10.5 litres of supernatant, previously concentrated by ultrafiltration then stored at -20°C before use and corresponding to 84 litres of starting broth, was chromatographed on phenyl Sepharose after adding 0.307 kg of NaCl (q.s. 0.5 M), then filtration on Durieux N°127 paper.

15 Analysis by SDS-PAGE of the supernatant of filtered culture thus obtained is shown in Figure 7 (well 3).

By using a Pharmacia XK 50/30 column provided with 500 ml of Phenyl Sepharose® Fast Flow High Substitution (Pharmacia) stored under ethanol at 25 %, then equilibrated with a solution of 0.5 M NaCl before use, concentration by chromatography on phenyl Sepharose was carried out in the following manner:

The concentrated salted supernatant obtained above (conductivity 40 mS.cm⁻¹) is applied to the column at a flow rate of 40 ml/mn. The column is then successively eluted at the same flow rate with 1.5 litres of 0.5M NaCl, then with 1.5 litres of Milli-Q water collecting the column effluent in fractions of 20 ml. The total proteins and the concentration of salts are detected as in Example 1.

30 The fractions corresponding to the peak of the protein eluted with water were analysed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA. The combined fractions containing the G-CSF (400 ml) contain 357 mg of GA-CSF titrated by HPLC corresponding to a yield of 67.9 % with a purity of 20.6 %. The solution of GA-GCSF phenyl Sepharose thus obtained has a conductivity of 2 mS.cm⁻¹ at 20°C.

The solution of phenyl Sepharose was also analyzed by SDS-PAGE (Figure 7, well 4). After stabilization by adding

Pefabloc (0.2 mg/ml) and benzamidine (1mM), the solution was immediately used for the following stage of chromatography on hydroxyapatite.

By using a Pharmacia XK 50/30 column provided with 290 g of hydroxyapatite Macro-Prep® Ceramic, Type II (Bio-Rad) previously suspended in 5 litres of 1 mM sodium phosphate buffer at pH 6 (1 mM buffer, pH 6), then equilibrated by percolation of 1 litre of 250 mM sodium phosphate buffer at pH 6 (250 mM buffer, pH 6) at a flow rate of 50 ml/mn, then by percolation of 5 litres of 1 mM buffer, pH 6 (thus generating a column of 500 ml of hydroxyapatite), chromatography on hydroxyapatite was carried out in the following manner:
400 ml of the solution of GA-GCSF of stabilised phenyl Sepharose obtained above is applied to the column of hydroxyapatite at a flow rate of 50 ml/mn. The column is then eluted with 1.50 litres of 1 mM buffer, pH 6 collecting the column effluent in fractions of 50 ml. The total proteins as well as the conductivity of the column effluent are detected as indicated in Example 1.

The fractions collected were analysed by analytical RP-HPLC and by ELISA. The combined fractions (400 ml) contain 331 mg of GA-GCSF titrated by HPLC corresponding to a chromatography yield of 92.5 % with a purity higher than the 90 % estimated by HPLC.

The solution of hydroxyapatite thus obtained was also analysed by SDS-PAGE (Figure 7, well 5).

Example 5: Subsequent purification of G-CSF after chromatography on hydroxyapatite.

The example illustrates the subsequent stages of purifying G-CSF which can be used after passing over hydroxyapatite in a multistage purification process.

From of a solution of hydroxyapatite of human GA-GCSF obtained according to the process of the invention, a stage of chromatography on a cation exchanger, then a stage of gel filtration chromatography were carried out in the following manner:

1) chromatography on a cation exchanger.

A Pharmacia XK 26/40 column provided with 170 ml of SP Sepharose® Fast Flow (Pharmacia) was equilibrated by washing at a flow rate of 13.2 ml/mn with 1.380 litres of Milli-Q water, then with 1.380 litres of 20 mM sodium acetate buffer at pH 5.3 (20 mM buffer, pH 5.3). 390 ml of hydroxyapatite solution of GA-GCSF obtained in Example 4 is applied to the column of SP Sepharose at a flow rate of 13.2 ml/mn. The column is then washed at the same flow rate with 414 ml of 20 mM buffer, pH 5.3, then with 1 litre of elution buffer corresponding to a gradient of NaCl, varying from 0 to 250 mM in 5 column volumes (850 ml) of the 20 mM buffer, pH 5.3 over 52 minutes, collecting the column effluent in fractions of 13.2 ml. By detection of the total proteins in the column effluent by absorption at 280 nm, the elution of a protein peak is observed. The fractions collected were analysed for their G-CSF content by analytical RP-HPLC chromatography and by ELISA. The combined fractions (237 ml) contain 255 mg of GA-GCSF titrated by HPLC corresponding to a yield of 78.8 % and a purity of 98.7 %.

The solution of SP Sepharose thus obtained was also analysed by SDS-PAGE (Figure 7, well 6). 2) gel filtration chromatography. 220 ml of the solution of SP Sepharose of GA-GCSF obtained above was concentrated approximately 10 times beforehand by ultrafiltration in a 300 ml Amicon cell provided with a PLGC membrane (Millipore), at + 4°C and under a nitrogen pressure of 2 bars. The UF concentrate thus obtained (21 ml) contains 253 mg of GA-GCSF titrated by HPLC corresponding to a yield of 107.1 %. The UF concentrate was also analysed by SDS-PAGE (Figure 7, well 7).

The UF concentrate was then subjected to a stage of gel filtration chromatography in the following manner: Two Pharmacia XK 26/40 columns, each provided with 150 ml of Superdex™ 200 prep grade (Pharmacia) and equilibrated by washing with 1.35 litres of Milli-Q water at a flow rate of 3.3 ml/mn, were set up in series, then equilibrated with 2.650 litres of PBS buffer (1X) at the same flow rate. 265 ml of Superdex™ 200 prep grade using two columns is thus

obtained.

10 ml of concentrated solution of SP Sepharose of GA-GCSF obtained above is applied to the sequence of columns at a flow rate of 3.3 ml/min. The columns are then washed at the same flow rate with 300 ml of PBS buffer (1X). By detection of the total proteins in the column effluent by absorption at 280 nm, the elution of a protein peak is observed. The collected fractions were analysed for their G-CSF content by analytical RP-HPLC chromatography. The combined fractions (42.9 ml) contain 85.3 mg of GA-GCSF titrated by HPLC corresponding to a yield of 70.7 % and a purity greater than 99 %.

Similarly, 10 ml of the concentrated solution of SP Sepharose of GA-GCSF obtained above is chromatographed by gel filtration under the conditions indicated above, but by using a 20 mM sodium acetate buffer, pH 5.5, Tween® 20 (0.005 %) in the place of the PBS buffer (1X). The combined fractions (42.9 ml) contain 95.4 mg of GA-GCSF titrated by HPLC corresponding to a yield of 79.1 % and a purity greater than 99 %.

Figure 7 shows the analysis by SDS-PAGE of the solution of filtration gel obtained in the PBS buffer (well 9) and in the acetate buffer, pH 5.5 (well 11) respectively.

CLAIMS

- 1) Process for purifying G-CSF from a biological sample comprising the stages of
 - a) reducing the volume of the biological sample containing
 - 5 G-CSF by hydrophobic-interaction chromatography in order to obtain a concentrated, desalted and enriched fraction,
 - b) passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound in order to obtain a concentrated, desalted and enriched fraction
 - 10 containing G-CSF and
 - c) collecting the G-CSF.
- 2) Process according to claim 1 in which the G-CSF collected has a purity of at least 90 %.
- 3) Process according to claim 1 in which the biological
- 15 sample is a cell culture supernatant.
- 4) Process according to claim 1 in which the G-CSF is a human G-CSF (h G-CSF).
- 5) Process according to claim 1 in which the volume reduction stage comprises placing of the biological sample on
- 20 a hydrophobic-interaction chromatography support of phenyl type under conditions allowing the fixation of the G-CSF, then its elution.
- 6) Process according to claim 5 in which the phenyl type support is Phenyl Sepharose®.
- 25 7) Process according to claim 6 in which fixation on Phenyl Sepharose® is carried out in a buffer with an ionic strength comprised between 0 and 60 mSi and elution is carried out by reducing the ionic strength or the salt concentration in the fixation buffer.
- 30 8) Process according to claim 6 in which the fixation on Phenyl Sepharose® is carried out in a buffer containing NaCl at a concentration comprised between 0.1 and 1 M.
- 9) Process according to claim 8 in which the fixation on Phenyl Sepharose® is carried out in a buffer containing NaCl
- 35 at a concentration comprised between 0.1 and 0.5 M and the elution is carried out with water.
- 10) Process according to claim 1 in which the stage of passing over hydroxyapatite is carried out in a buffer of

ionic strength comprised between 2 and 30 mSi and at a pH comprised between 5.5 and 7.5.

11) Process according to claim 10 in which the buffer comprises phosphate at a concentration comprised between 1
5 and 10 mM.

12) Process according to claim 10 in which the buffer is a 1 mM phosphate buffer and the pH is comprised between 6.0 and 7.5.

13) Process for purifying G-CSF which can be included in a
10 multistage process for purifying G-CSF from a biological sample comprising the stages of

- a) reducing the volume of the biological sample containing the G-CSF by hydrophobic-interaction chromatography in order to obtain a concentrated, desalted and enriched fraction,
- 15 b) passing the concentrated fraction over hydroxyapatite under conditions where the G-CSF is weakly bound in order to obtain a concentrated, desalted and enriched fraction containing G-CSF and
- c) collecting the G-CSF.

20 14) Process according to claim 13 in which the multistage process moreover comprises one or more stages of chromatography chosen from the group constituted by ion exchange, reversed phase gel filtration, or affinity chromatography.

25 15) Process for eliminating the contaminating proteins from a solution containing G-CSF and the contaminating proteins comprising

- a) passing the solution over hydroxyapatite by which the contaminating proteins are fixed to the hydroxyapatite and
30 the G-CSF is weakly bound and
- b) elution of the G-CSF.

16) Process according to claim 15 in which the elution of the G-CSF is carried out by simple washing with the fixation buffer.

35 17) Process according to claim 15 in which the solution containing the G-CSF is prepared by hydrophobic-interaction chromatography of a biological sample containing G-CSF.

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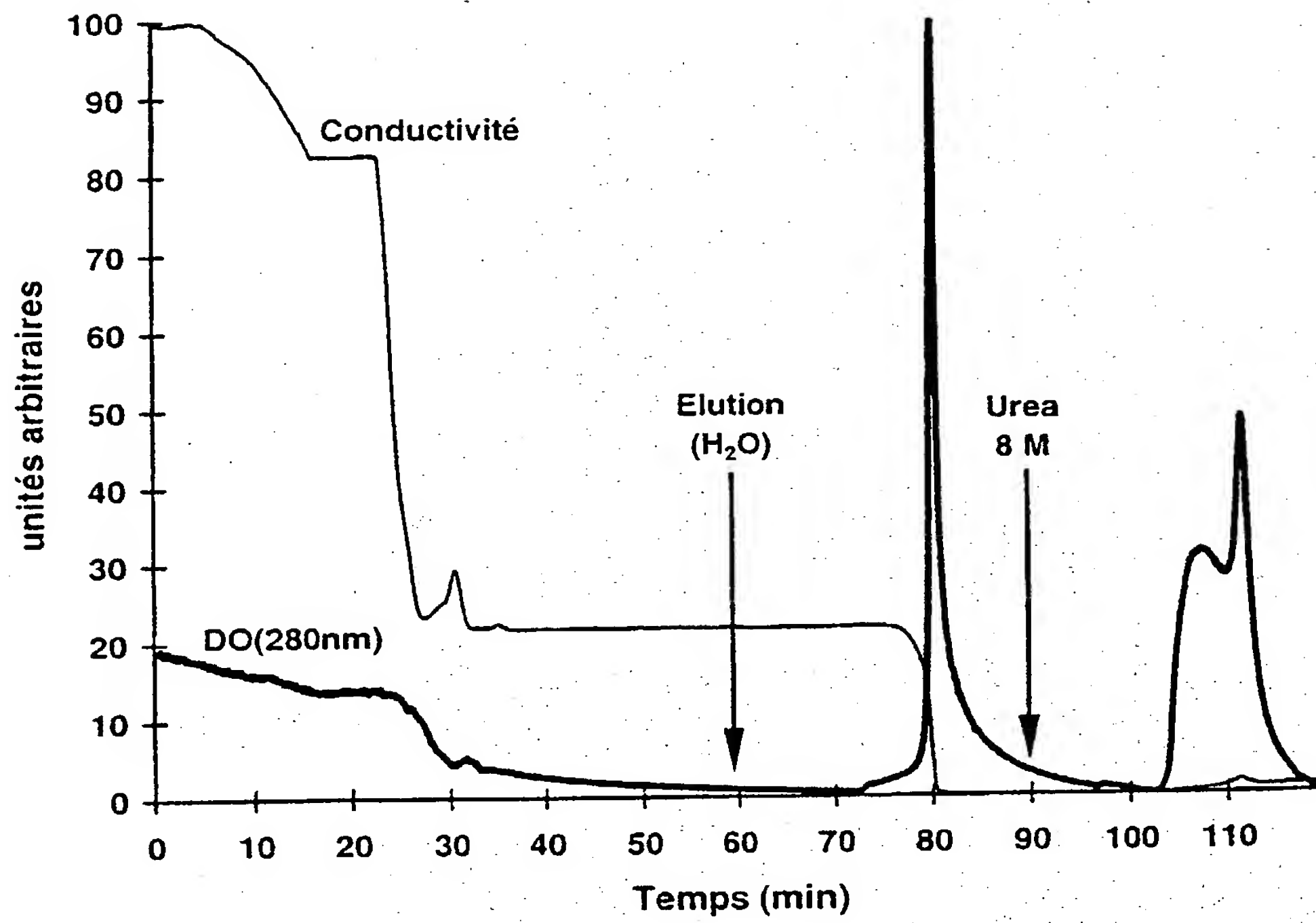
Phenyl-Sepharose

FIGURE 1

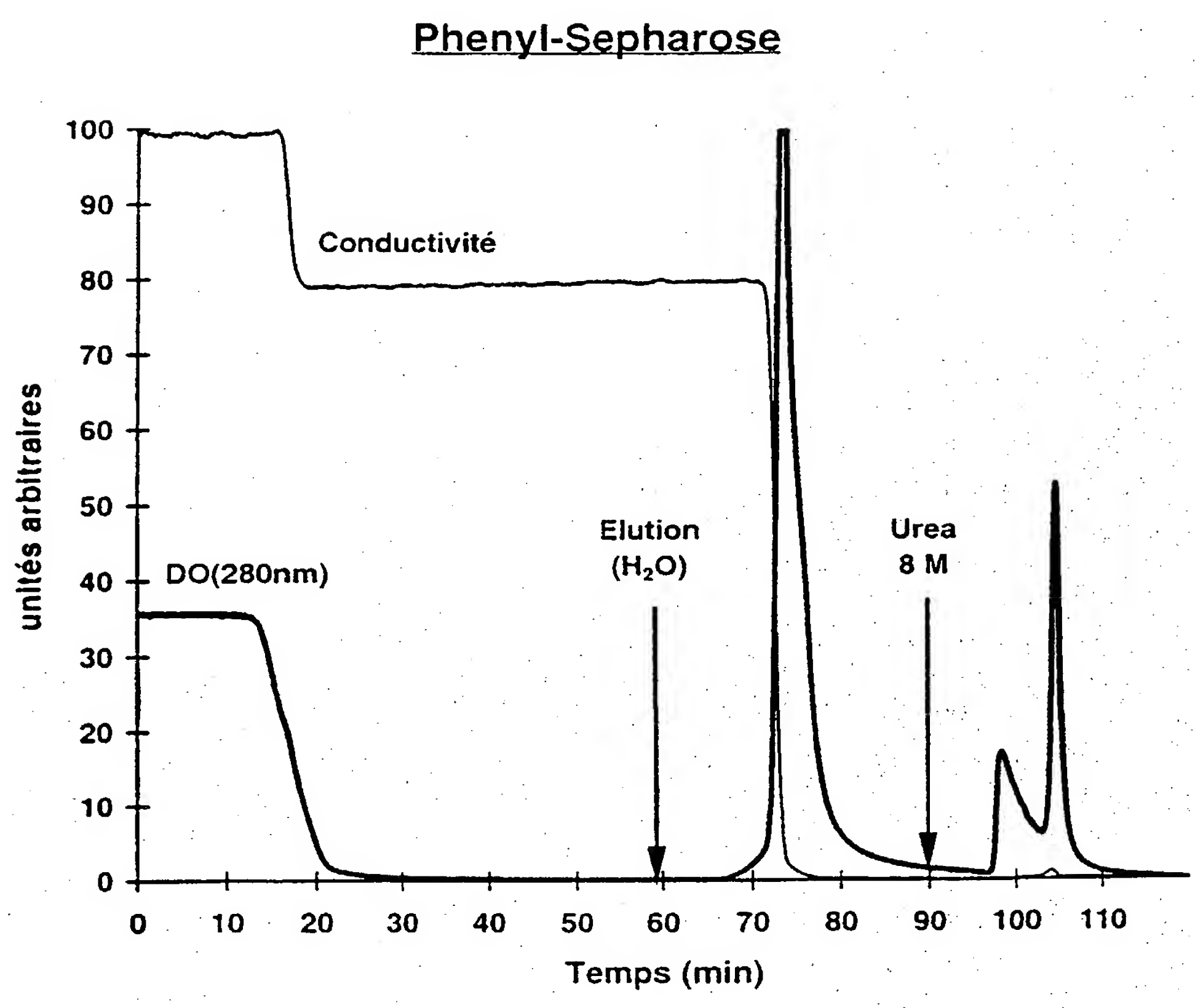


FIGURE 2

Hydroxyapatite

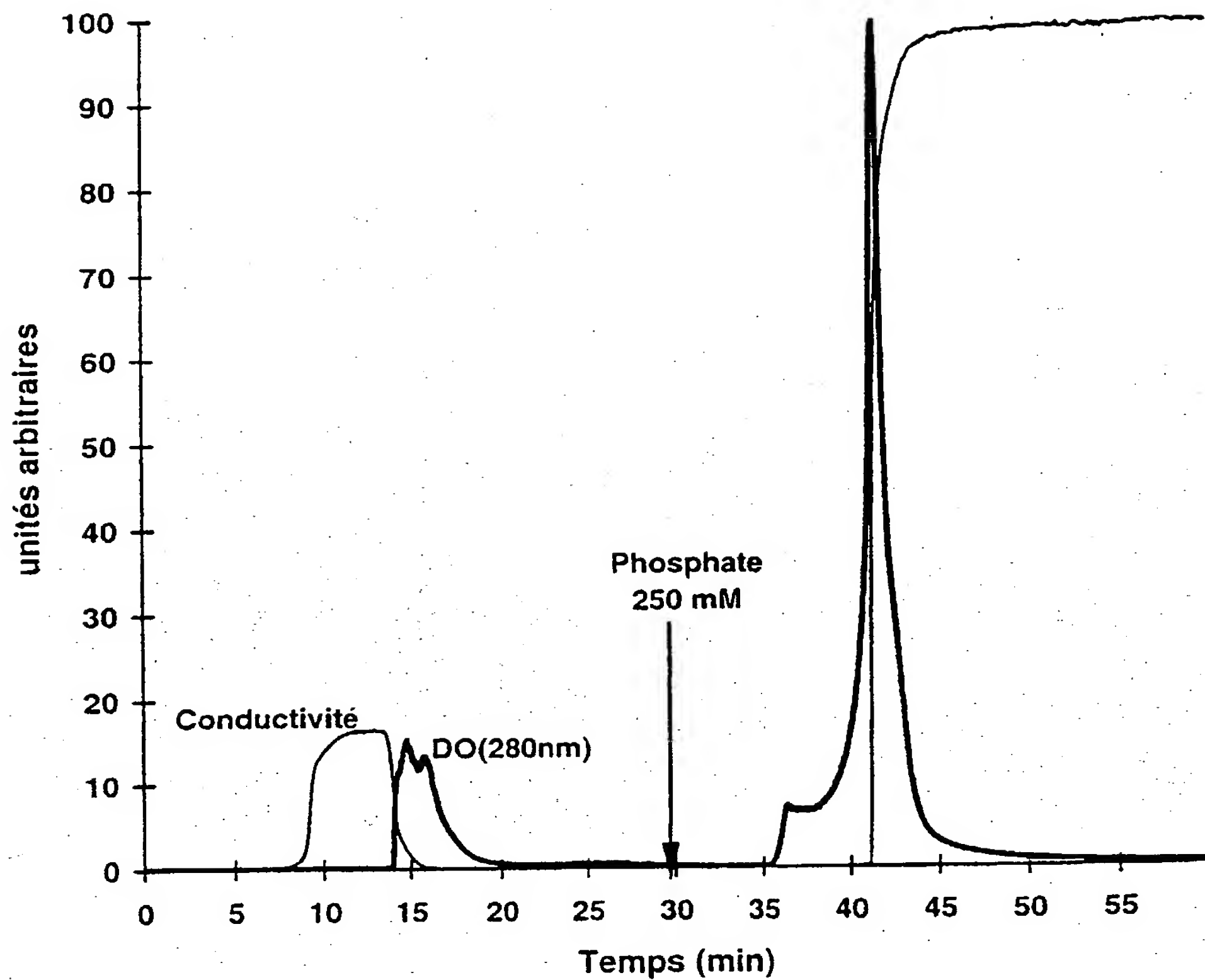


FIGURE 3

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DO(214nm)

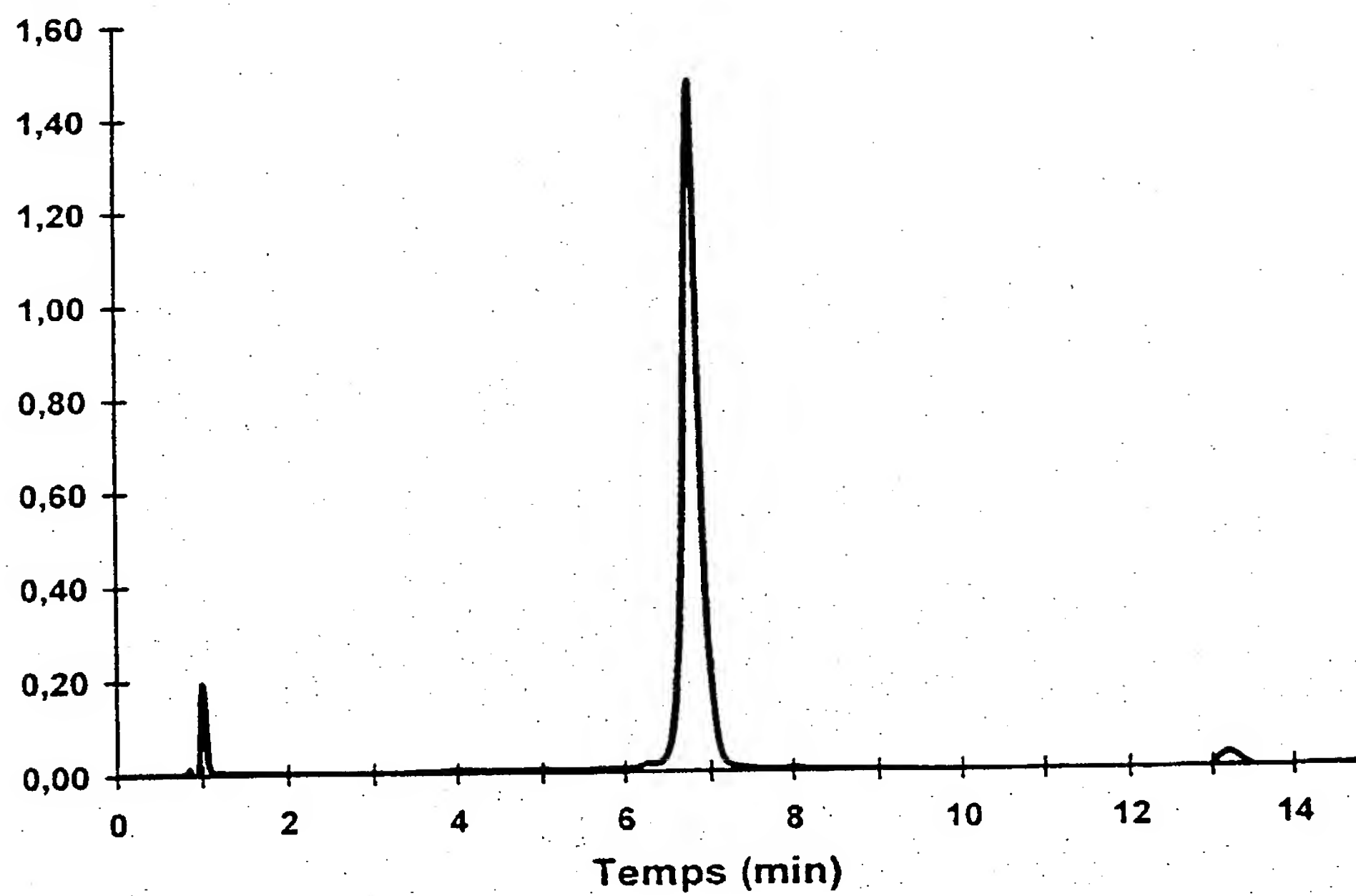


FIGURE 4

Hydroxyapatite

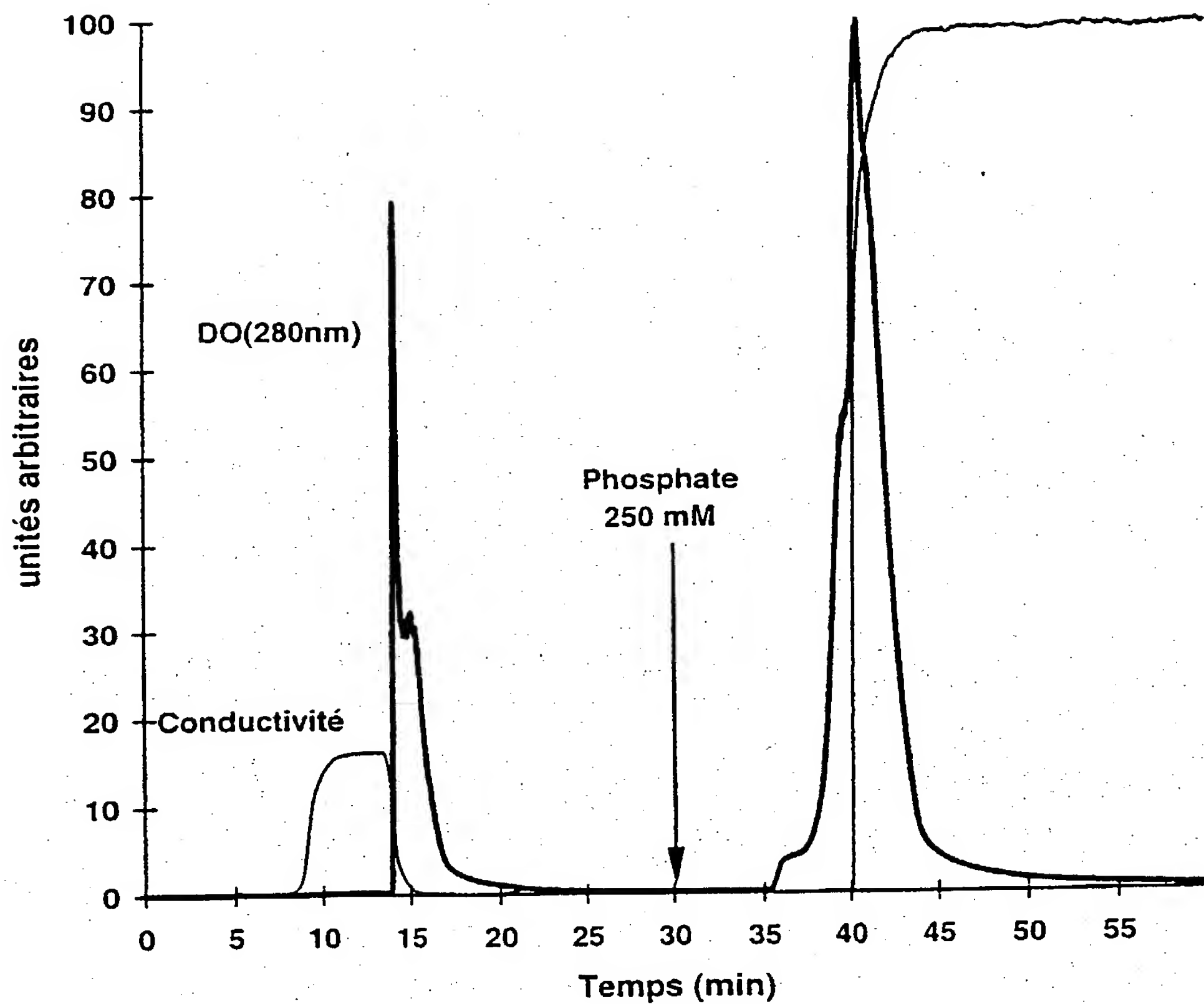


FIGURE 5

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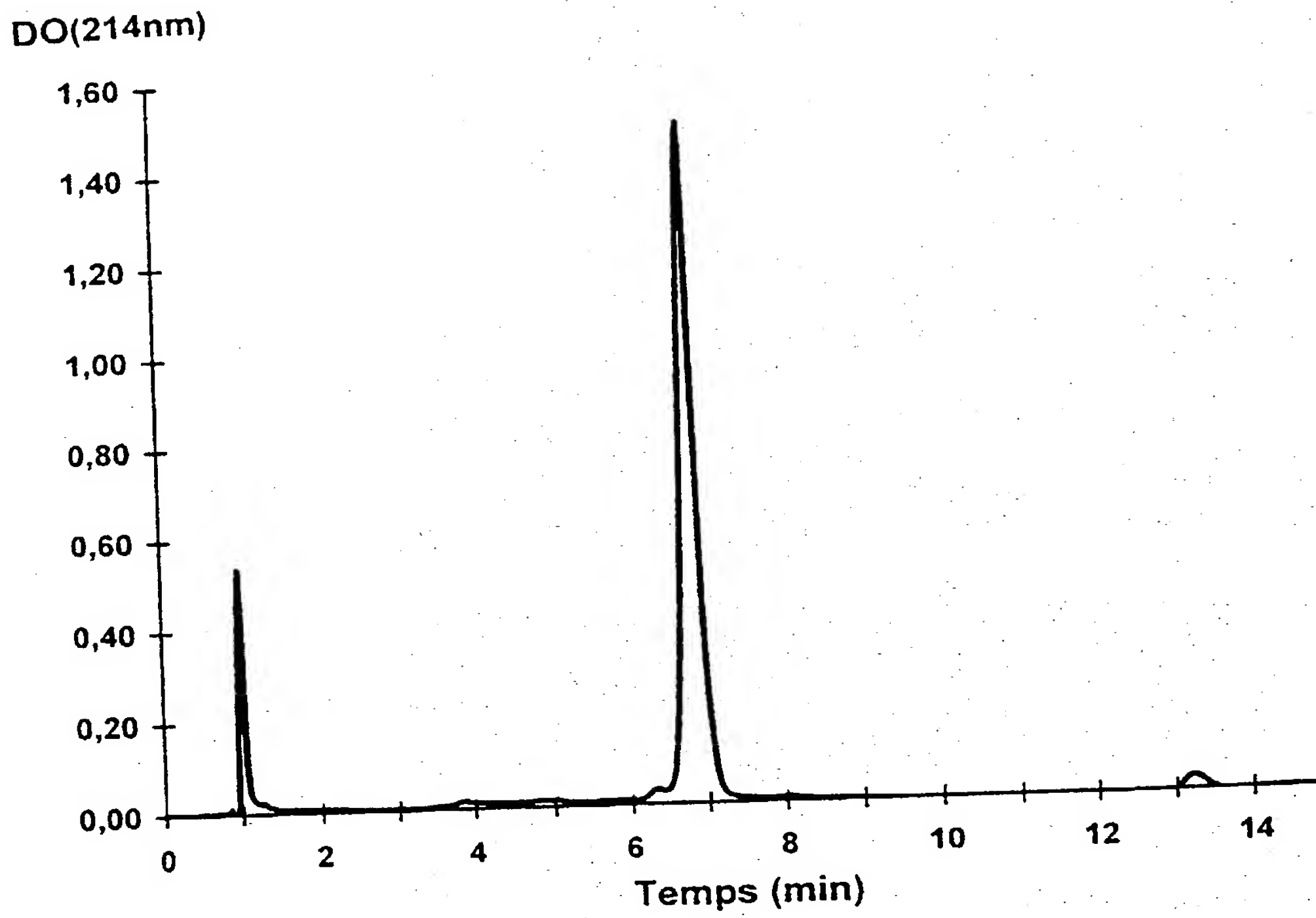


FIGURE 6

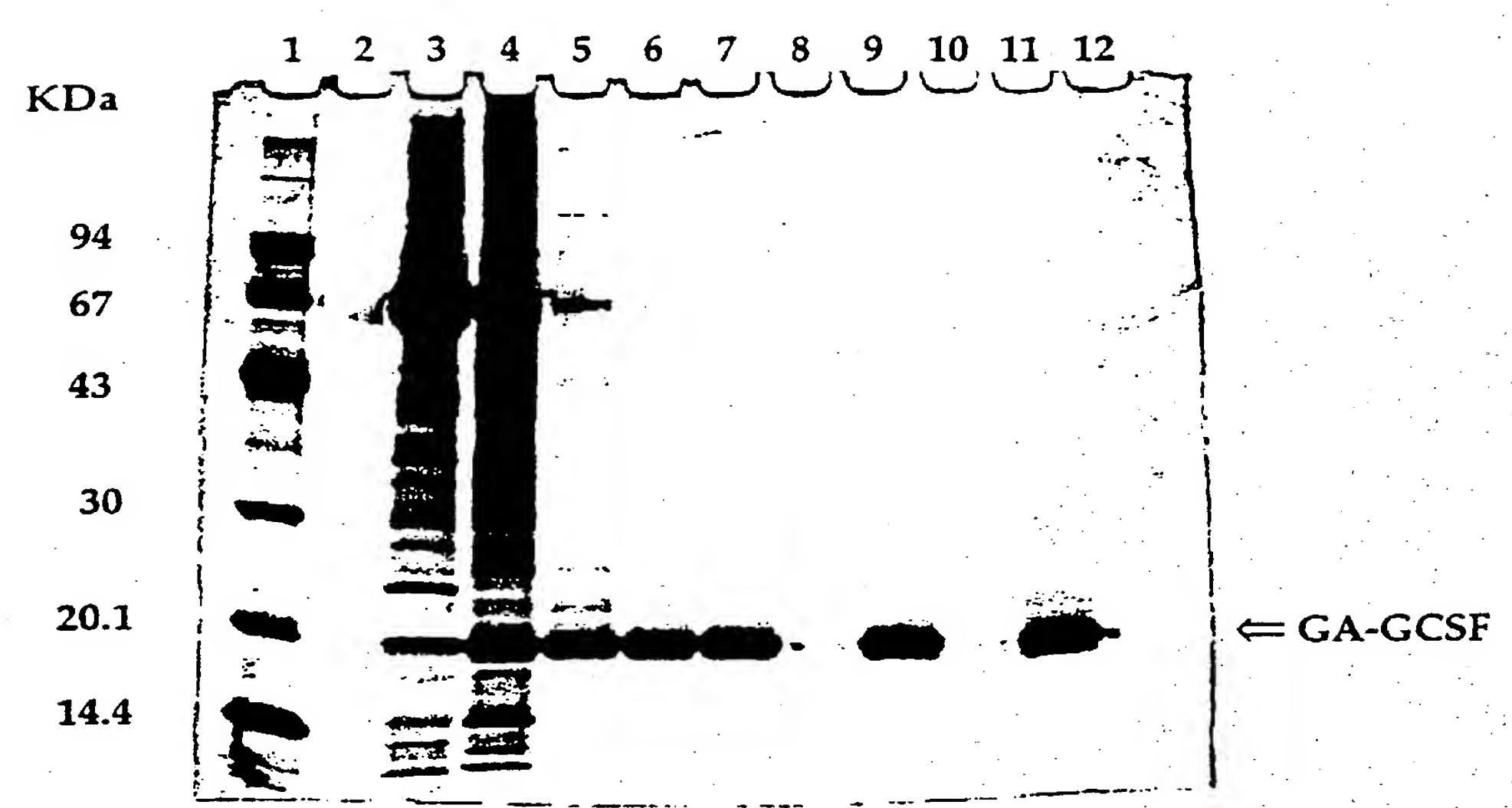


FIGURE 7

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **METHOD FOR PURIFYING GRANULOCYTE COLONY STIMULATING FACTOR**, the application for which was filed on January 7, 2002, under attorney docket number 03806.0531, and which was amended by submission of a Substitute Specification and Preliminary Amendment on January 7, 2002.

I hereby state that I have reviewed and understand the contents of this application, including the written description and claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application(s) designating at least one country other than the United States, listed below and have also identified below, any foreign application(s) for patent or inventor's certificate, or any PCT International application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119
France	99/08831	July 8, 1999	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application(s) and the national or PCT International filing date of this application:

Application Number	Date of Filing	Status (Patented, Pending, Abandoned)
PCT/FR00/01937	July 6, 2000	Pending

I hereby appoint the following attorney and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. **FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.**, Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings, IV, Reg. No. 20,645; Jerry D. Voight, Reg. No. 23,020; Laurence R. Hefter, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,098; Herbert H. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,610; Michael C. Elmer, Reg. No. 25,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson, Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,680; Dennis P. O'Reilly, Reg. No. 27,932; Allen M. Sokal, Reg. No. 26,695; Robert D. Bajefsky, Reg. No. 25,387; Richard L. Stroup, Reg. No. 28,478; David W. Hill, Reg. No. 28,220; Thomas L. Irving, Reg. No. 28,619; Charles E. Lipsey, Reg. No. 28,165; Thomas W. Winland, Reg. No. 27,605; Basil J. Lewris, Reg. No. 28,818; Martin I. Fuchs, Reg. No. 28,508; E. Robert Yoches, Reg. No. 30,120; Barry W. Graham, Reg. No. 29,924; Susan Haberman Griffen, Reg. No. 30,907; Richard B. Racine, Reg. No. 30,415; Thomas H. Jenkins, Reg. No. 30,857; Robert E. Converse, Jr., Reg. No. 27,432; Clair X. Mullen, Jr., Reg. No. 20,348; Christopher P. Foley, Reg. No. 31,354; John C. Paul, Reg. No. 30,413; Roger D. Taylor, Reg. No. 28,992; David M. Kelly, Reg. No. 30,953; Kenneth J. Meyers, Reg. No. 25,146; Carol P. Einaudi, Reg. No. 32,220; Walter Y. Boyd, Jr., Reg. No. 31,738; Steven M. Anzalone, Reg. No. 32,095; Jean B. Fordis, Reg. No. 32,984; Barbara C. McCurdy, Reg. No. 32,120; James K. Hammond, Reg. No. 31,964; Richard V. Burgujian, Reg. No. 31,744; J. Michael Jakes, Reg. No. 32,824; Thomas W. Banks, Reg. No. 32,719; Christopher P. Isaac, Reg. No. 32,616; Bryan C. Diner, Reg. No. 32,409; M. Paul Barker, Reg. No. 32,013; Andrew Chanhon Sonu, Reg. No. 33,457; David S. Forman, Reg. No. 33,694; Vincent P. Kovalick, Reg. No. 32,867; James W. Edmondson, Reg. No. 33,871; Michael R. McGurk, Reg. No. 32,045; Joann M. Neth, Reg. No. 36,363; Gerson S. Panitch, Reg. No. 33,751; Cheri M. Taylor, Reg. No. 33,216; Charles

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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